THE INTERSTITIAL CELLS OF CAJAL OF THE EQUINE GASTROINTESTINAL TRACT: DEVELOPMENT AND DISEASE

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DECLARATION

I hereby certify that this thesis and the work it describes was written and performed by myself. Some of the material discussed in this thesis has been presented at meetings and/or published. Reprints are included in Appendix 5 of this thesis.

CONSTANZE FINTL

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ABSTRACT

Gastrointestinal motility disorders constitute a substantial problem in the horse both in terms of welfare and economic cost. It is often difficult to identify the underlying cause as many horses recover spontaneously or with empirical medical treatment. Recently, the gastrointestinal pacemaker cells, the interstitial cells of Cajal (ICC), were identified in the horse. These cells initiate and coordinate gastrointestinal motility patterns through the generation of slow waves. This current study investigated the ICC in the equine intestine both in health and disease using immunohistochemical, electrophysiological and molecular biological techniques. The aim of these studies was to further our knowledge on the role of ICC in equine intestinal motility disorders.

Using immunohistochemistry targeting a receptor tyrosine kinase, c-Kit, of the ICC, the ontogeny of these cells in the horse was described. This demonstrated a proximal to distal, as well as a transmural developmental gradient in the large intestine with evidence of ongoing postnatal development. Additionally, the density of ICC in healthy, adult horses was compared to that in horses with obstructive intestinal disease requiring surgical correction. This demonstrated a significant reduction in ICC density in horses with obstructive disorders of the large intestine compared to the control group. In addition, ICC density and distribution was investigated in recovered chronic equine grass sickness horses as well as in normal and diseased donkeys.

The c-*kit* gene, encoding the c-Kit receptor of the ICC, was identified in intestinal tissue samples. The transcription levels of this gene were determined and comparisons made

between healthy and diseased horses using quantitative real-time PCR analysis. A parallel immunohistochemical assessment was also performed. These studies demonstrated no significant changes in gene transcription levels, although a reduction in ICC density (using c-Kit immunohistochemistry) in horses with an obstructive disorder of the large colon was evident, suggesting that future investigations of c-*kit* post-transcriptional control as well as c-Kit protein pathology are warranted.

Investigation of the *in vitro* electrical activity of the equine large colon was carried out using intracellular microelectrode recording techniques in order to characterise slow waves and other electrical activities in this anatomical region from normal and diseased horses.

It is hoped that this study will help improve our knowledge of the involvement of ICC in equine intestinal motility in health and disease. Furthermore, it may facilitate future studies investigating the involvement and function of the ICC in the equine gastrointestinal tract.

ABBREVIATIONS

ACh	Acetylcholine
ANS	Autonomic nervous system
CGRP	Calcitonin gene-related peptide
CM	Circular muscle
cDNA	Complimentary deoxyribose nucleic acid
dNTP	deoxynucleotidetriphosphates
DNA	Deoxyribose nucleic acid
EGS	Equine grass sickness
ENS	Enteric nervous system
ESP	External submucous plexus
EtBr	Ethidium bromide
GDNF	Glial cell line-derived neurotropic factor
GI	Gastrointestinal
H&E	Haematoxylin and eosin
5-HT	5-Hydroxytryptamine
ICC	Interstitial cells of Cajal
ICC-DMP	Interstitial cells of Cajal-deep muscular plexus
ICC-IM	Interstitial cells of Cajal-intramuscular
ICC-MP_{SB}	Interstitial cells of Cajal-myenteric plexus small bowel
ICC-MP_{C}	Interstitial cells of Cajal-myenteric plexus colon
ICC-SM	Interstitial cells of Cajal-submucosal border
IP_3	Inositol 1,4,5 triphosphate
ISP	Internal submucous plexus
LSB	Long spike bursts
MMC	Migrating myoelectrical complexes
MP	Myenteric plexus
MPOs	Myenteric potential oscillations
NA	Noradrenaline
NANC	Non-adrenergic non-cholinergic
NO	Nitric oxide
NOS	Nitric oxide synthase
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PGP 9.5	Protein gene product 9.5
PI3-kinase	Phosphatidylinositol 3'-kinase
RMP	Resting membrane potential
RNA	Ribose nucleic acid
RPM	Rounds per minute
RT	Reverse transcriptase
RT-PCR	Reverse transcriptase- polymerase chain reaction
SCF	Stem cell factor
SMP	Submucosal plexus
SSS	Short spike bursts
VIP	Vasoactive intestinal peptide

1 INTRODUCTION

1.1 Normal intestinal function in the horse

The horse is a monogastric herbivore with a hindgut specialised for fibre digestion which provides a major energy source through the production of volatile fatty acids by microbial activity (Lopes and Pfeiffer 2000). The passage of material through the stomach and small intestine prior to its arrival in the caecum and colon may also have some important effects on fermentative digestion (Herdt 1992). This includes exposure of plant particles to gastric acid, which may increase their susceptibility to microbial attack, as well as digestion and absorption of some sugars and starches (Herdt 1992).

For maximum efficiency of further digestion, the horse's hindgut has evolved to create an environment favourable for microbial development and fibre digestion. This includes the expansion of the caecum and large colon to effectively create large digestion chambers (Figure 1), as well as having adapted motility patterns in order to further optimise digestion (Argenzio 1975). The to-and-fro movements (haustrum-to-haustrum exchanges) in the caecum and the retropulsive movement near the pelvic flexure are two of these adapted mechanisms (Dyce and Hartman 1973; Argenzio *et al.* 1974; Sellers *et al.* 1982a, 1984). The latter ensures a delay in the transit of ingesta through the large intestine in order to optimise microbial action and nutrient absorption (Argenzio *et al.* 1974; Argenzio 1975; Sellers *et al.* 1982a).

However, the evolved complex motility patterns, in association with specialised anatomical arrangements such as the large caecum and the narrow lumen of the pelvic flexure are thought to be involved in the pathophysiology of some types of colic in the horse (Argenzio 1975; Sellers *et al.* 1982b; Lopes and Pfeiffer 2000).

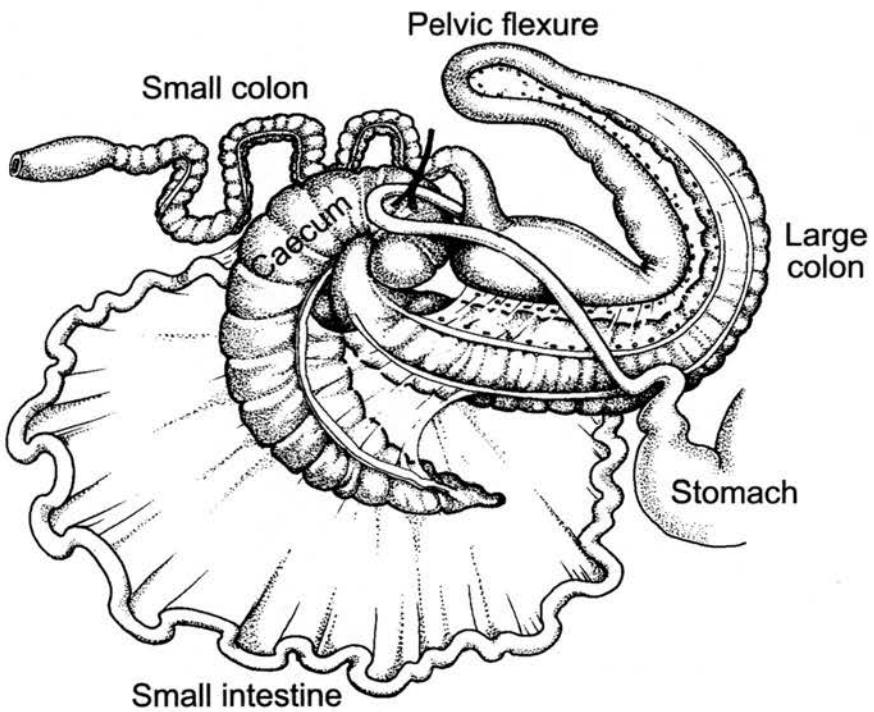


Figure 1: The intestinal tract of the adult horse (adapted from Dyce *et al.* 1987).

The term "colic" refers to abdominal pain that may be caused by a number of different problems. In horses, most of these problems involve the gastrointestinal (GI) tract, but diseases of other structures within or associated with the abdomen such as the kidneys, liver, uterus, and peritoneum may also result in signs of colic (Traub-Dargatz *et al.* 2001). For the purposes of discussion in this thesis, the term colic will refer to abdominal pain only associated with GI disease.

1.2 Equine intestinal motility disorders

Gastrointestinal motility disorders constitute a significant cause of morbidity and mortality in the horse, as well as representing significant annual economic losses to the horse industry (Tinker *et al.* 1997a; Traub-Dargatz *et al.* 2001). Traub-Dargatz *et al.* (2001) estimated the annual cost of colic in the United States to be $115,300,000.

Other studies have demonstrated the incidence of colic and estimated subsequent fatality rates, as well as making an attempt to identify risk factors associated with this problem on a local and national scale (Proudman 1991; Cohen *et al.* 1995; Cohen and Peloso 1996; Reeves *et al.* 1996; Kaneene *et al.* 1997; Tinker *et al.* 1997a, b; Cohen *et al.* 1999; Hillyer *et al.* 2001; Traub-Dargatz *et al.* 2001; Hillyer *et al.* 2002). Some of these studies also highlighted the large proportion of colic episodes that remain undiagnosed primarily because they resolve spontaneously or with empirical medical treatment such as analgesic administration (Proudman 1991; Tinker *et al.* 1997a; Traub-Dargatz *et al.* 2001).

1.2.1 Disorders of the equine intestinal tract

The association between many of the motility disorders seen in the horse and certain risk factors may be of help when attempting to make a clinical diagnosis. However, this may still present a significant problem mainly due to the large equine abdomen and limited possibilities to physically evaluate all abdominal structures.

Small intestinal disorders may have an acute and violent presentation particularly if distension and/or ischemia of the intestine are involved. Examples of this include strangulating lipomas, epiploic foramen entrapment, ileal impactions, or more uncommonly mesenteric rents where small intestine becomes entrapped and subsequently strangulated in this defect (Sembrat 1975; Parks *et al.* 1989; White 1990; Blikslager *et al.* 1992; Edwards and Proudman 1994; Gayle *et al.* 2000; Little and Blikslager 2002). Other disorders of the small intestine may involve abnormal motility patterns without a physical obstruction. This includes conditions such as equine grass sickness (EGS) (Cottrell *et al.* 1999), inflammatory conditions such as anterior enteritis and inflammatory bowel disease (Platt 1986), although the latter may present as an obstructive problem following the formation of circumferential mural bands (Scott *et al.* 1999). Worm infestation can also be a contributing factor as tapeworm infection of the terminal ileum has been associated with ileal impactions as well as spasmodic colic of other parts of the intestinal tract (Proudman *et al.* 1998). Similarly, caecocaecal and caecocolic intussusceptions have been reported as a possible complication of larval cyathostomiasis (Mair *et al.* 2000).

Disorders of the large intestine may also have an acute and violent presentation and are again typically associated with distension and/or ischemia. This often involves a rotation of the large colon where the degree of rotation influences the severity of clinical signs as well as the degree of vascular compromise (Hackett 1983; Johnston and Freeman 1997). The rotations seen include left or right dorsal displacements and large colon volvulus although only the latter is associated with severe vascular compromise (Johnston and Freeman 1997).

The most common form of large colon obstructions not associated with rotation is impaction with ingesta (White 1990). Typically this occurs where the intestinal lumen becomes narrower such as the pelvic flexure but can occur in all areas (White 1990; White and Dabareiner 1997). The underlying causes of all these conditions are often unknown although both management factors such as feeding as well as intrinsic motility problems have been implicated (White 1990; White and Dabareiner 1997; Schusser *et al.* 2000).

As seen from this brief overview, a number of intestinal conditions may occur in the horse with some being more common than others. In order to better understand their aetiology, it is important to obtain epidemiological as well as functional information. A number of epidemiological studies have been carried out in recent years to help identify risk factors associated with equine colic. A summary of some of these findings is described in the following section.

1.2.2 Factors involved in equine motility disorders

Based on the epidemiological data available obtained from a number of studies, it seems reasonable to conclude that colic is a multifactorial problem involving many variable and fixed risk factors. Variable risk factors include management practices such as feeding and husbandry as well as exercise intensities. Conflicting results concerning the importance of each risk factor have been reported which could be the result of different study designs as well as regional variation in the sample population. Tinker *et al.* (1997b) demonstrated a marked dose-relationship between the total amount of concentrates consumed and an increased risk of colic. This study also demonstrated that horses were at a reduced risk of developing colic if they were fed whole grain feed compared to horses that were not (Tinker *et al.* 1997b). These findings were the exact opposite to those of Reeves *et al.* (1996), although the latter investigation involved a different study population and design. A recent change in diet was found to be significantly associated with colic in a number of other studies (Proudman 1991; Cohen *et al.* 1995; Cohen and Peloso 1996; Cohen *et al.* 1999; Hudson *et al.* 2001a).

Studies into the relationship between colic incidence and anthelmintic treatment have also produced conflicting results. Recent anthelmintic administration was found to be associated with a reduced risk of colic in one study (Hudson *et al.* 2001a), whereas in others it was associated with an increased risk of colic (Proudman 1991; Kaneene *et al.* 1997; Cohen *et al.* 1999; Hillyer *et al.* 2002). However, in a study by Cohen *et al.* (1995), no association between recent administration of an anthelmintic and colic was

shown. These differences could be due to different study designs and the management programme of the sample population. Proudman *et al.* (1998) demonstrated that tapeworm (*Anoplocephalata perfoliata*) infection was a significant risk factor for spasmodic colic and colic associated with ileal impaction in the horse. The involvement of both small (cyathostomes) and large strongyles (*Strongylus vulgaris*) in equine colic has also been well documented (Duncan and Pirie 1972; Uhlinger 1990; Love 1992; Reid *et al.* 1995).

Studies into the relationship between exercise and colic have indicated that there is a higher incidence of colic in horses on a higher level of exercise or with a recent change in exercise intensity (Love *et al.* 1994; Cohen *et al.* 1995; Kaanene *et al.* 1997; Tinker *et al.* 1997a; Cohen *et al.* 1999; Hillyer *et al.* 2001; Hillyer *et al.* 2002). Only two of these studies related the colic incidence to a specific intestinal condition (pelvic flexure impaction and colonic obstruction respectively) (Love *et al.* 1994; Hillyer *et al.* 2002), again highlighting that a large proportion of reported colic episodes are undiagnosed.

Associations between colic incidence and fixed risk factors have also been investigated. There appears to be an increased risk of colic in the "middle age-group" (2-10 years) (Tinker *et al.* 1997b) and with increasing age (mean age 10.3 years) (Kaneene *et al.* 1997) when compared to younger animals. There is also an association of age with specific conditions such as pedunculated lipomas in older animals, especially older ponies (Sembrat 1975; Blikslager *et al.* 1992; Edwards and Proudman 1994), while

animals in the two to seven years of age range appear to be at particular risk for developing EGS (Doxey *et al.* 1991).

In spite of sometimes conflicting information from these studies, there appears to be agreement on the increased risk of colic in horses with a history of a previous episode of colic where this was investigated (Ducharme *et al.* 1983; Cohen *et al.* 1995; Reeves *et al.* 1996, Cohen *et al.* 1999, Tinker *et al.* 1997a, b; Hillyer *et al.* 2002). Animals with recurrent episodes of colic represent a common diagnostic challenge (Mair and Hillyer 1997b). Mair and Hillyer (1997b) noted that obstructive disorders represented a large proportion of recurrent and prolonged colics in their study population, and it was proposed that these animals may have been suffering from an underlying intrinsic motility problem.

Two studies have demonstrated a significant reduction in myenteric neuronal densities in horses with acute and chronic obstructive disorders of the caecum and large colon (Schusser and White 1997; Schusser *et al.* 2000). It was proposed that these changes may have been a reason for the increased risk of recurrent colic from colonic or caecal dysfunction in these horses (Schusser *et al.* 2000). Recently, a significant reduction in the gastrointestinal pacemaker cells, the interstitial cells of Cajal (ICC), was detected in horses with EGS and it was proposed that these changes may be a contributing factor to the motility disorders observed in these animals (Hudson *et al.* 2001b).

In order to understand the possible implications of all these proposed factors, it is necessary to have an understanding of how normal intestinal motility patterns are generated and of the components involved.

1.3 Components involved in generating and maintaining normal intestinal motility patterns.

At the most basic level, the intestinal components involved in generating and maintaining normal intestinal motility patterns are the enteric nervous system (ENS), the intestinal smooth muscle layers, and the interstitial cells of Cajal (ICC). Each of these components is essential to this process and will be described in detail.

1.3.1 Enteric nervous system

Motility of a particular segment of intestine is influenced by input from the intrinsic ENS as well as by extrinsic input from the autonomic nervous system (ANS) (Furness and Costa 1987). The extrinsic sympathetic innervation occurs primarily via postganglionic sympathetic fibres whose cell bodies are in the prevertebral and paravertebral ganglia (Furness and Costa 1987). Some sympathetic fibres synapse on neurones of the intrinsic ENS in the submucosal and myenteric plexuses, whereas others exert a direct effect on GI muscles and glands (Herdt 1992). The neurotransmitter of these postganglionic sympathetic fibres is noradrenaline (NA) (Herdt 1992). Parasympathetic innervation is provided primarily by the preganglionic branches of the

vagus nerve which all terminate in the ganglia of the intramural plexuses to synapse with the enteric neurones that innervate the smooth muscle and secretory cells of the GI tract (Furness and Costa 1987). Acetylcholine (ACh) is the neurotransmitter agent between the preganglionic parasympathetic fibres and the intrinsic neurones of the ENS (Herdt 1992). In spite of the extensive extrinsic nervous input to the GI tract, if the sympathetic and parasympathetic nerves are cut, many of the motor and secretory activities continue to function because of the intrinsic control by the ENS (Berne and Levy 1993).
The ENS consists of the myenteric and submucosal plexuses and in the small intestine, also the deep muscular plexus (Furness and Costa 1980) (Figure 2).

Interneurones in these plexuses connect intrinsic afferent sensory fibres with efferent neurones to smooth muscle and secretory cells as well as receiving input from the ANS (Herdt 1992; Berne and Levy 1993). The myenteric plexus is concerned primarily with motility, while the submucosal plexus is involved in regulating transepithelial transport, mucosal blood flow and secretomotor functions (Furness and Costa 1987).

The myenteric nerve plexus is located between the two muscle layers (circular and longitudinal) of the *muscularis externa* (Irwin 1931). It consists of ganglia containing neuronal cell bodies of different sizes as well as bundles of unmyelinated fibres that interconnect the ganglia (Irwin 1931). In adult guinea pigs the ganglia in the myenteric plexus under the taenial bands of the large intestine were found to be larger and more circular in shape than the ganglia between these bands (Irwin 1931). This study also noted that the neuronal cell count in this area of the large intestine was markedly greater

than in the regions between the taenial bands. High neuronal counts were also demonstrated in the taenial bands of the left and right ventral and dorsal colon of the horse although only one horse was examined (Burns 1992). Burns and Cummings (1991) noted in an earlier study that there was an increased neuronal density in the myenteric plexus region of the pelvic flexure region compared to other areas of the caecum and large colon in 23 normal horses. This corresponded reasonably well with a subsequent study by Schusser and White (1997) who demonstrated increased myenteric plexus neurone densities of the pelvic flexure and left dorsal and transverse colons compared to the right dorsal, left ventral, and right ventral colons of 15 clinically normal horses.

The submucosal plexus is positioned adjacent to the mucosa and its neurones are primarily concerned with the control of transepithelial ion transport (Cooke 1994) and mucosal blood flow (Surprenant 1994). It consists of two interconnected plexuses, the internal submucous plexus (ISP) and the external submucous plexus (ESP), in several species including the horse (Gunn 1968; Scheuermann *et al.* 1989; Pearson 1994; Balemba *et al.* 1999). Pearson (1994) noted a considerable variability in the form and size of the ganglia within these two plexuses in the equine jejunum. However, no obvious structural differences between the two were evident as individual nerve cell bodies were of comparable diameter and the mean number of cells per ganglion in each plexus was very similar (Pearson 1994). Different functional roles for these two plexuses in the pig have been proposed based on the differences in distribution and occurrence of neurotransmitters as well as neurone types in the two layers (Timmermans *et al.* 1990;

Thomsen *et al.* 1997). It was proposed that the ISP could be involved in the control of physiological processes such as secretion, absorption and blood flow while the ESP could primarily serve a sensory function (Thomsen *et al.* 1997).

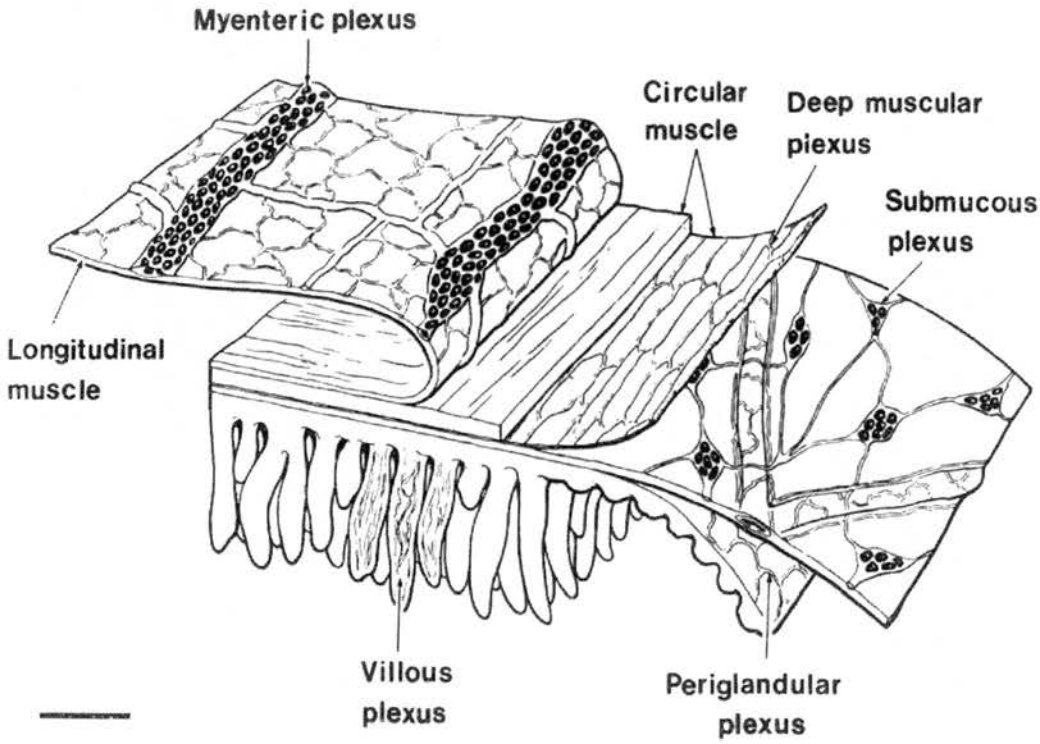


Figure 2: Schematic diagram of the equine small intestine demonstrating the different muscle layers of the *muscularis externa* as well as the nerve plexuses of the enteric nervous system (adapted from Furness and Costa 1980).

More than 20 neuroregulatory substances have been identified in the GI tract although the number is likely to be much higher (Furness and Costa 1987). Both the extrinsic parasympathetic as well as some efferent intrinsic neurones releases ACh (Furness and Costa 1987). Generally, these cholinergic neurones are excitatory, increasing muscle contractions by bringing the resting membrane potential closer to threshold through opening of sodium (Na^+) channels (Furness and Costa 1987; Herdt 1992; Berne and Levy 1993). However, it has also been suggested that ACh may cause relaxation of the circular muscle of the equine jejunum when the muscle is at a higher level of tone by stimulating the release of the inhibitory neurotransmitter nitric oxide (NO) (Malone *et al.* 1999).

Both NA and adrenaline generally have an inhibitory effect on intestinal motility (Furness and Costa 1987; Berne and Levy 1993). Their effect is mediated either directly on smooth muscle, or indirectly through inhibiting ACh release from intrinsic cholinergic neurones (Furness and Costa 1987). The distribution and the effect of stimulation of the different sympathetic receptor types appear to vary between different anatomical locations in the equine small intestine (Malone *et al.* 1996; Re *et al.* 1997; Malone *et al.* 1999).

The ENS also releases non-adrenergic non-cholinergic (NANC) neuromodulatory substances (Furness and Costa 1987; Herdt 1992; Berne and Levy 1993). Combined adrenergic and cholinergic blockade has revealed the likely involvement of intrinsic NANC neurotransmitters in equine jejunal function (Malone *et al.* 1999; Rakestraw *et*

al. 2000). Another study looked at the distribution of various NANC neuropeptides in the equine jejunum, caecum and colon and found that the regional distribution of immunoreactive neuronal elements was uniform for substance P, methionine-enkephalin and calcitonin gene-related peptide (CGRP), but not for vasoactive intestinal peptide (VIP) (Burns and Cummings 1993). VIP showed the greatest regional variation, being slightly more plentiful at the pelvic flexure and left dorsal colon (Burns and Cummings 1993). Pearson (1994) noted the presence of VIP as well as galanin and neuropeptide Y in nerve cell bodies and fibres of each of the plexuses in the equine jejunum. Generally, VIP has an inhibitory effect on intestinal motility through progressive inhibition ahead of giant migrating contractions, essential for rapid propulsion of a large bolus (Cortesini *et al.* 1995).

Apart from VIP, the other main NANC inhibitory transmitter of the intestinal tract is NO (Sanders and Ward 1992) which is synthesised from L-arginine, catalysed by the enzyme nitric oxide synthase (NOS) (Moncada *et al.* 1991). Rather than causing complete suppression of intestinal motility, it appears to modulate activity through its basal release (Keef *et al.* 1997). Studies in the equine intestinal tract have largely supported an inhibitory role for NO (Rakestraw *et al.* 1996; Malone *et al.* 1999; Rakestraw *et al.* 2000; Van Hoogmoed *et al.* 2000). The main inhibitory effect in the jejunum appeared to arise in the circular muscle layer (Rakestraw *et al.* 1996; Malone *et al.* 1999) while in the ventral colon it was also present in the taenial bands (Van Hoogmoed *et al.* 2000).

Substance P is one of the main NANC initiators of intestinal contraction and belongs to a group of neuropeptides called tachykinins (Furness and Costa 1987). It too has been found in enteric nerves in the equine intestine (Burns and Cummings 1993; Pearson 1994; Sonea *et al.* 1997). The specific effects of substance P are determined by the subtype of receptor present in the target tissue (Belloli *et al.* 1994). There are three known subtypes of tachykinin receptors, distinguished by their relative affinities for substance P and other tachykinins (Belloli *et al.* 1994). It appears that in the equine small intestine, the neurokinin 2 receptor predominates (Belloli *et al.* 1994; Sonea *et al.* 1997). This appeared not to be the case in the equine colon, although it was not possible to establish which tachykinin receptor was the most predominant (Belloli *et al.* 1994; Sonea *et al.* 1997).

Both extrinsic and intrinsic sensory neurones use CGRP as a neurotransmitter (Grider 1994). This peptide is released from extrinsic sensory fibres in response to muscle stretch and from intrinsic sensory fibres following mucosal stimulation (Grider 1994). The release from intrinsic sensory fibres is dependent on release of 5-hydroxytryptamine (5-HT) (Foxx-Orenstein *et al.* 1996). 5-HT is present in mucosal enterochromaffin cells as well as in a small population of interneurones that project caudally within the myenteric plexus (Foxx-Orenstein *et al.* 1996). It is thought that mucosal stimulation will result in release of 5-HT from the enterochromaffin cells to activate sensory neurones that mediate both motor and secretory reflexes (Foxx-Orenstein *et al.* 1996). The 5-HT receptor has also been identified in the equine intestinal tract where

stimulation of some of the receptor subgroups resulted in increased force of intestinal contraction (Nieto *et al.* 2000; Weiss *et al.* 2002).

In most cases, the enteric neurones contain more than one putative transmitter or neuromodulator, and in many instances co-release of more than one substance at enteric nerve endings may occur (Furness and Costa 1987). The combination of neuroactive substances present in a particular neurone correlates with the morphology and function of that neurone and with its projections (Furness and Costa 1987).

1.3.2 Smooth muscle

Gastrointestinal smooth muscle cells are closely electrically coupled and hence function as a contractile unit rather than as single cells (Berne and Levy 1993). These units of intestinal smooth muscle cells exhibit rhythmic, depolarisations and repolarisations of the membrane potential called slow waves which are initiated by the ICC (Ward *et al.* 1994; Huizinga *et al.* 1995; Ward *et al.* 1995). The passive conduction of slow waves from the ICC depolarise smooth muscle cells and activates different populations of ionic conductances. Further response of the smooth muscle cells may be manifest as either a small, nonregenerative enhancement in depolarisation (i.e. an apparent increase in slow wave amplitude), or calcium (Ca^{2+}) action potentials superimposed upon the slow waves (Horowitz *et al.* 1999). Critical to the generation of action potentials is the opening of Ca^{2+} channels of which the L-type channels are necessary for excitation-contraction coupling (Horowitz *et al.* 1999). Most smooth muscle cells also express T-type Ca^{2+}

channels, although these activate at more negative potentials than the L-type channels and inactivate rapidly at more depolarised voltages, and hence are not involved in the contraction-coupling mechanism (Farrugia 1999). Once an electrical threshold is reached, with an associated action potential and smooth muscle contraction, the cell repolarises partly through inactivation and deactivation of Ca^{2+} channels, but primarily through opening of a variety of potassium (K^+) channels including delayed rectifier and Ca^{2+} activated large conductance channels (Bolton *et al.* 1999; Farrugia 1999).

Neural inputs can condition the smooth muscle cell response to slow waves. Release of excitatory neurotransmitters activates nonselective cation channels and increases the effectiveness of slow waves to bring the smooth muscle membrane potential to threshold (Horowitz *et al.* 1999; Koh *et al.* 2003). Release of inhibitory neurotransmitters activates K^+ channels, which decrease the probability of reaching threshold (Horowitz *et al.* 1999; Koh *et al.* 2003).

The activity of intestinal smooth muscle cells and ICC are clearly closely linked. However, blockade of L-type Ca^{2+} channels does not block the generation of slow waves by ICC, suggesting that other inward current sources are available in ICC and needed to initiate these events (Horowitz *et al.* 1999). This will be discussed further in the next section.

1.3.3 Interstitial cells of Cajal

Both ICC and smooth muscle cells are influenced by nervous input creating a closely integrated network of motility control as reviewed by Sanders (1996). This thesis will focus in particular on the role ICC play in not only initiating and maintaining normal intestinal motility patterns, but also their possible involvement in horses with intestinal motility disorders.

1.4 The role and function of the interstitial cells of Cajal

1.4.1 Broad overview and history

Santiago Ramon y Cajal (1893, 1911) first described the interstitial cells more than a century ago as small fusiform or stellate cells with prominent nuclei and varicose processes that formed networks in GI tissues. He suggested that these cells were primitive neurones, although this has since been the subject of much discussion because of their ultrastructure and anatomical location. Other investigators proposed different functions for the cells including them being primitive or specialised smooth muscle cells or fibroblasts (Taxi 1952, 1965; Cook and Burnstock 1976). However, this debate could probably never be settled by anatomical location or ultrastructural analysis alone, because the morphological features of ICC are similar to several cell types within the *muscularis externa* including both smooth muscle cells and fibroblasts and especially in the developing intestinal tract where these cell types are difficult to differentiate (Thuneberg 1982; Faussone-Pellegrini 1984, 1987; Faussone-Pellegrini *et al.* 1985,

1996). However, the focus of attention changed as the functional role of the ICC as pacemaker cells became apparent. This was largely a result of developmental studies based on the discovery that ICC express a receptor tyrosine kinase, c-Kit (Ward *et al.* 1994; Huizinga *et al.* 1995; Torihashi *et al.* 1995). These studies followed the discovery by Maeda *et al.* (1992) that blocking of the c-Kit-receptor with neutralizing antibodies resulted in a reduction of c-Kit-immunoreactive cells, proposed to be ICC, as well as abnormal contractile patterns of intestinal muscles with loss of slow wave activity. Earlier dissection experiments had already established the areas of origin of slow wave activity within the GI tract which varied with the anatomical location. In the small intestine, pacemaker activity was found to arise from the border between the circular and longitudinal muscle layers in the myenteric plexus region (Hara *et al.* 1986; Suzuki *et al.* 1986). The colon appeared to exhibit a more complex electrical pattern, although dissection and ablation studies suggested that the submucosal aspect of the circular muscle was the primary pacemaker site (Christensen *et al.* 1969; Durdle *et al.* 1983; Smith *et al.* 1987a, b). It has also been demonstrated that a second pacemaker site exists in the myenteric plexus region creating so-called myenteric potential oscillations (MPOs) thought to be the primary "pacesetter potentials" of the outer longitudinal muscle layer (Smith *et al.* 1987b; Plujà *et al.* 2001). The MPOs also spread into the circular muscle layer where they summate with slow waves and it was proposed that the degree of summation determines the force of contraction in this muscle layer (Smith *et al.* 1987b).

ICC are also thought to be involved in mediating neurotransmission as well as active propagation of electrical events. The initial suggestion that ICC may be involved in neurotransmission was based primarily on morphological studies (Thuneberg 1982; Daniel and Posey Daniel 1984; Rumessen *et al.* 1993). The frequent close contacts formed between varicosities of the nerve endings and ICC (in many cases <25nm) was highly suggestive of an important role for ICC in neurotransmission (Thuneberg 1982; Daniel and Posey Daniel 1984; Rumessen *et al.* 1993; Daniel *et al.* 1998; Wang *et al.* 1999, 2000). It was further suggested that ICC are functionally interposed between NANC inhibitory nerves and smooth muscle cells and hence play an important role in enteric inhibitory neurotransmission (Huizinga *et al.* 1990; Publicover *et al.* 1993). Ward *et al.* (1998) confirmed this by demonstrating that ICC were involved in mediating inhibitory neurotransmission in the lower oesophageal and pyloric sphincters in mice. More specifically, this involved the facilitation of NO-dependent inhibitory neurotransmission in these areas. It was also suggested that parallel innervation of ICC located in the deep muscular plexus area of the small intestine (ICC-DMP) and smooth muscle cells by enteric inhibitory neurones exists (Ward *et al.* 1998). The deep muscular plexus area is a rich neuronal network located between the outer and the thinner inner muscle layers of the small intestine (Gabella 1994; Kobilo *et al.* 2003). This connection had already been proposed by Daniel and Posey-Daniel (1984) in an earlier morphological study. Wang *et al.* (1999, 2000) noted that the ICC-DMP and ICC in the circular muscle of the colon (ICC-IM) were heavily innervated by not only inhibitory, but also excitatory motor neurones. Epperson *et al.* (2000) provided further support for

these findings by identifying receptors for both inhibitory and excitatory neurotransmitters on ICC in the stomach and small intestine of mice.

It has also been suggested that ICC may not only mediate but also amplify neural inputs in the canine colon (Publicover *et al.* 1993). Exogenous NO caused an increase in intracellular Ca^{2+} concentrations, which may signal ICC to increase NO production, producing amplification of inhibitory neural signals (Publicover *et al.* 1993). This concept was supported by showing the expression of a constitutive NOS in ICC of the canine proximal colon indicating the capacity for NO synthesis within these cells (Xue *et al.* 1994).

ICC are also thought to play a role in the active propagation of slow waves in the gastrointestinal tract because these can be propagated over relatively large distances (Sanders *et al.* 1990; Horiguchi *et al.* 2001; Koh *et al.* 2003). In order for slow waves to persist over such distances, there must be a mechanism of active propagation and this appears to be an exclusive property of ICC networks (Koh *et al.* 2003). It has been further suggested that slow waves of longitudinal and circular muscle are synchronised through ICC (Taylor *et al.* 1977; Liu *et al.* 1998).

All the functions described above are thought to be carried out by separate groups of ICC (Sanders 1996). In a comprehensive review, Sanders (1996) proposed that ICC located in the myenteric plexus region of the small bowel (ICC-MP_{SB}) were involved in generating pacemaker activity, while cells located primarily in the deep muscular plexus

region of the circular muscle layer (ICC-DMP) were involved in mediating neurotransmission. As described above, the colon appears to have a more complex arrangement, although it is likely that ICC near the submucosal border (ICC-SM) generate the primary pacemaker activity with a second site being present in the myenteric plexus region (ICC-MP_C). In addition, intramuscular ICC of the colon (ICC-IM_C) are thought to play a role in mediating neurotransmission. However, it is likely that there are several classes of ICC whose function is not yet fully understood (Sanders 1996).

1.4.2 Ontogeny

The majority of current knowledge on the development of ICC is based on studies primarily in the mouse, rat and chicken (Torihashi *et al.* 1995; Lecoin *et al.* 1996; Young *et al.* 1996; Torihashi *et al.* 1997; Klüppel *et al.* 1998). There have also been a number of human clinical studies demonstrating that ICC, or precursors of ICC, start to colonise the intestinal tract at an early stage of foetal development (Kenny *et al.* 1998a, b; 1999; Wester *et al.* 1999). Generally, this seems to occur about a third to half-way through gestation, with some variation between species (Lecoin *et al.* 1996; Young *et al.* 1996; Torihashi *et al.* 1997; Kenny *et al.* 1999; Wester *et al.* 1999). No previous studies have investigated the development of these cells in the equine GI tract.

The identification of ICC was made possible through the use of the c-Kit receptor as an immunohistochemical marker (Ward *et al.* 1994; Huizinga *et al.* 1995; Torihashi *et al.*

1995; Ward *et al.* 1995). Prior to this, developmental studies were difficult to perform as definitive identification of these cells required ultrastructural analysis (Thuneberg 1982; Faussone-Pellegrini 1984, 1987; Faussone-Pellegrini *et al.* 1985, 1996; Rumessen *et al.* 1993). Additionally, immature ICC possess few of the ultrastructural features associated with mature ICC, making this process very difficult (Faussone-Pellegrini *et al.* 1996). The use of the c-Kit receptor as an ICC marker has also allowed for earlier identification of these cells in their development and indeed much earlier than had been suggested from ultrastructural studies alone (Faussone-Pellegrini *et al.* 1985; Maeda *et al.* 1992; Lecoin *et al.* 1996; Young *et al.* 1996; Torihashi *et al.* 1997; Ward and Sanders 2001).

The embryological origin of the ICC has been the subject of much interest. Cajal (1911), as well as other authors (Boeke 1949; Dupont and Sprinz 1964), proposed that these cells were primitive neurones. However, it has since been established that ICC are mesenchymal in origin (Lecoin *et al.* 1996; Young *et al.* 1996; Klüppel *et al.* 1998), rather than being derived from the migrating neural crest cells as initially hypothesised. The original study to determine this was carried out by Lecoin *et al.* (1996) using a quail-chick marker system. Chimeric bowels, in which the enteric nerves were quail in origin, were produced by grafting quail vagal neural crest into chick embryos at embryonic day 2 (E2), i.e. before the colonisation of chick neural crest cells. All c-Kit-positive cells observed later were of chick origin, as determined by *in situ* hybridisation, demonstrating that the quail vagal neural crest cells were not involved in the ICC development (Lecoin *et al.* 1996). To further support this conclusion, the same authors also found that ultrastructurally typical ICC developed in aneural chick intestine cultured

on chorio-allantoic membranes. Thus, the development of ICC was independent of enteric neurones. A similar conclusion was reached in studies on the developing murine colon where segments of colon removed from embryos prior to colonisation of neural crest cells were cultured under the renal capsule of host adult mice (Young *et al.* 1996). This showed that after 18-41 days, c-Kit-immunoreactive ICC were present in the aneuronal explants (Young *et al.* 1996). In support of this, a later study using glial cell line-derived neurotrophic factor (GDNF) knockout mice which lack enteric neurones throughout most of the gut, found that these animals developed normal ICC in spite of this abnormality (Ward *et al.* 1999). Additionally, a separate study demonstrated that c-Kit-positive ICC precursor cells do not express c-*ret*, a neural crest cell marker (Torihashi *et al.* 1997).

In contrast, it has been suggested that ICC and smooth muscle cells share a common developmental origin (Torihashi *et al.* 1997; Klüppel *et al.* 1998). There are two main arguments for this. Firstly, in the late murine embryonic stage (E15-17), cells expressing c-Kit aggregated into clusters at the outer aspect of the small intestine, peripheral to the developing myenteric plexus (Torihashi *et al.* 1997). Some of these c-Kit-immunopositive cells were also immunopositive for vimentin or desmin, both smooth muscle markers (Torihashi *et al.* 1993, 1994). By birth, the outer longitudinal smooth muscle cells lost expression of vimentin and became strongly desmin positive as the adult phenotype developed, while the ICC retained vimentin expression into adulthood (Torihashi *et al.* 1997; Klüppel *et al.* 1998). Secondly, Torihashi *et al.* (1999) found that

blockade of c-Kit signalling in neonatal mice induced transdifferentiation of ICC into a smooth muscle phenotype in the small intestine of neonatal mice.

The role of c-Kit in ICC development has been subject to much investigation. Ward *et al.* (1995) suggested that embryonic activation of c-Kit is important because blockade of c-Kit impaired ICC development in the murine GI tract. These observations agreed with previous findings using mutant mice (Ward *et al.* 1994; Huizinga *et al.* 1995). Mutations that completely block the function of c-Kit are fatal and homozygotes (*W/W*) usually die *in utero* (Russel 1979). Most investigations of animals carrying this mutation have therefore been performed on heterozygotes (W/W^{V}) (Ward *et al.* 1994; Huizinga *et al.* 1995). The W^{V} mutation is a point mutation at amino acid 660 that reduces, but does not abolish, the tyrosine kinase function of c-Kit (Nocka *et al.* 1990). These studies noted that the number of ICC in the myenteric region was markedly reduced disrupting the integrity of the network of cells (Ward *et al.* 1994; Huizinga *et al.* 1995).

c-Kit signalling also appears to be important for ICC development after birth in the murine intestine (Torihashi *et al.* 1995; Klüppel *et al.* 1998). Multiple injections of neutralizing c-kit antibodies (ACK2) administered to mice between birth and day 8 resulted in a significant reduction in the number of c-Kit-immunoreactive cells, proposed to be ICC, in the small intestine (Maeda *et al.* 1992). A similar study which also included the murine colon was carried out by Torihashi *et al.* (1995) confirming these earlier findings, although at this point, it had been established that the c-Kit immunoreactive cells were indeed ICC (Huizinga *et al.* 1994; Ward *et al.* 1994).

Additionally, it was demonstrated that c-Kit antibody administration in neonatal mice was accompanied by altered contractile and electrical patterns with loss of electrical rhythmicity in the small intestine and reduced neural responses in the small intestine and colon (Torihashi *et al.* 1995; Klüppel *et al.* 1998). It was further noted that delaying administration of the c-Kit antibody until the animals were 20-30 days old required a much longer administration time before the myenteric ICC were affected (up to 35 days) (Klüppel *et al.* 1998). This suggests that at least the myenteric ICC may develop resistance to loss of c-Kit signalling with age (Klüppel *et al.* 1998).

The natural ligand for c-Kit is stem cell factor (SCF) or *Steel* factor (Zsebo *et al.* 1990). The c-Kit receptor has extracellular, membrane spanning, and cytoplasmic domains (Yarden *et al.* 1987). It is the extracellular domains that contain the receptor for SCF (Yarden *et al.* 1987). Binding of SCF activates the tyrosine kinase function of the extracellular domain (Rottapel *et al.* 1991). This results in autophosphorylation of tyrosine residues and binding of phosphatidylinositol 3'-kinase (PI3) and phospholipase C-γ1 (Rottapel *et al.* 1991).

As for the W^V mice, *Steel* mutant mice have disrupted small intestine ICC networks in the myenteric plexus region with corresponding absence of normal electrical slow wave activity (Ward *et al.* 1995; Mikkelsen *et al.* 1998). In other words, a defect of either receptor or ligand will result in abnormal ICC development and function in this anatomical region. Interestingly, both studies noted that the ICC in the deep muscular

plexus area of the small intestine appeared normal (Ward *et al.* 1995; Mikkelsen *et al.* 1998). It was suggested that there might be variable degrees of dependence or sensitivity toward the SCF/c-Kit pathway between different ICC classes (Ward *et al.* 1995). Consistent with this suggestion, Torihashi *et al.* (1997) noted that ICC in the deep muscular plexus region developed separately and later than ICC in the myenteric plexus region.

The source of SCF is still subject to some debate. SCF exists in two forms, soluble and membrane bound (Flanagan *et al.* 1991). It appears that it is the membrane bound form that is important for c-Kit signalling as *Steel* mutant mice are still able to produce the soluble form but not the membrane bound form (Flanagan *et al.* 1991). This implies that cells in the immediate vicinity of c-Kit must supply SCF. It has been reported that both smooth muscle cells and neurones in the human gastrointestinal tract express membrane bound SCF (Lammie *et al.* 1994). As ICC develop normally in mice lacking enteric neurons (Young *et al.* 1996; Ward *et al.* 1999) it is likely that smooth muscle cells may be an important source of SCF.

The onset of electrical rhythmicity and slow waves occurs after the development of ICC networks (Torihashi *et al.* 1997; Ward *et al.* 1997). However, there appears to be anatomical and inter-species variation regarding the exact timing of onset (Torihashi *et al.* 1997; Ward *et al.* 1997). Broadly speaking, within 7 days of birth, normal resting membrane potentials and electrical patterns are present throughout the GI tract in the species examined (Torihashi *et al.* 1997; Ward *et al.* 1997). These studies have also

shown that concomitant with abnormal ICC morphology, numbers or function, there is a corresponding lack of normal electrical patterns in the intestinal tract providing further evidence for the role of the ICC as pacemaker cells (Ward *et al.* 1994; Huizinga *et al.* 1995; Torihashi *et al.* 1995; Ward *et al.* 1995; Torihashi *et al.* 1999).

1.4.3 Generation of pacemaker activity and smooth muscle cell response

The exact mechanism of slow wave generation in the ICC is still not fully determined. Earlier studies proposed that this event was initiated through spontaneous activation of low-threshold, T-type Ca^{2+} channels which caused depolarisation of the resting membrane potential activating L-type Ca^{2+} channels leading to amplification of the current (Sanders 1996; Farrugia 1999). Other work has suggested that this pacemaker current is initiated by voltage-independent, Ca^{2+}-inhibited, non-selective cationic conductance in the ICC (Koh *et al.* 1998; Thomsen *et al.* 1998; Koh *et al.* 2002; Koh *et al.* 2003; Sanders *et al.* 2004). It is thought that Ca^{2+} release from inositol 1,4,5 triphosphate (IP_3) receptor-operated stores in the sarcoplasmic reticulum stimulates Ca^{2+} uptake into mitochondria via a Ca^{2+}-dependent transporter (Ward *et al.* 2000; Kim *et al.* 2002). Mitochondrial Ca^{2+}-uptake transiently reduces Ca^{2+} activity in the space close to the plasma membrane and this result in the activation of a Ca^{2+}-inhibited, non-selective cation conductance (Kim *et al.* 2002). It is thought that activation of these channels generates the pacemaker current (Kim *et al.* 2002; Koh *et al.* 2002). In other words, there appears to be a close association between IP_3 receptor-dependent Ca^{2+} stores, mitochondria and ion channels in the plasma membrane. In support of this theory, it has

been demonstrated that slow waves are abolished in mice lacking IP_3-R1 receptors (Suzuki *et al.* 2000).

Once the current is generated, it will travel across gap junctions from the ICC to smooth muscle cells and establish the slow wave depolarisation. If the slow wave amplitude is sufficient, voltage-dependent Ca^{2+} channels (dihydropyridine-sensitive L-type Ca^{2+} channels) are activated causing a contractile response (Horowitz *et al.* 1999). Addition of nifedipine, an L-type Ca^{2+} channel blocker, results in the cessation of contractile activity (Farrugia 1999). This has also been demonstrated experimentally in the horse in a study by Hudson *et al.* (2001c) who found that while nifedipine abolished the spiking contractile activity of the smooth muscle, it did not abolish the slow waves although their temporal characteristics were altered.

Other voltage-dependent channels, such as K^+ channels, tune the response of the smooth muscle cells and facilitate or prohibit the development of Ca^{2+}-dependent action potentials (spikes) (Horowitz *et al.* 1999; Koh *et al.* 2003). A variety of K^+ channel-blocking drugs can dramatically affect the pattern of electrical activity in GI muscles (Horowitz *et al.* 1999).

The response of intestinal smooth muscle to the ICC-generated pacemaker current is also strongly influenced by neural inputs (Daniel and Posey-Daniel 1984; Publicover *et al.* 1993; Daniel *et al.* 1998; Ward *et al.* 1998; Wang *et al.* 1999, 2000). Release of excitatory neurotransmitters activates non-selective cation channels and increases the

effectiveness of slow waves to bring the muscle cells to threshold (Horowitz *et al.* 1999). Release of inhibitory transmitters activates K^+ channels, which decrease the probability of the smooth muscle cells reaching threshold (Horowitz *et al.* 1999). All these findings emphasise that the initiation and maintenance of normal intestinal motility patterns is an integrated process involving not only ICC but also smooth muscle cells and a functional nervous network

Currently, the majority of investigations into equine GI motility patterns have been *in vitro* pharmacological studies evaluating muscle responses to various external stimuli or intrinsic nerve stimulation using isometric force transducers (Murray *et al.* 1994; Malone *et al.* 1996; Rakestraw *et al.* 1996; Malone *et al.* 1999; Nieto *et al.* 2000; Van Hoogmoed *et al.* 2000), as well as *in vivo* extracellular recordings of intestinal myoelectrical activity (Sellers *et al.* 1981; Roger and Ruckebusch 1987; Ruckebusch and Roger 1988; Merritt *et al.* 1989a, b; Lester *et al.* 1998; Merrit *et al.* 1998). Recently, *in vitro* intracellular microelectrode recordings of the equine jejunum and ileum have also been carried out in order to characterise electrical patterns and their response to some pharmacological compounds (Rakestraw *et al.* 2000; Hudson *et al.* 2001c, 2002). Hudson *et al.* (2001c) characterised slow wave frequencies and patterns in the equine ileum and found that the ileum displays, on average, 9 waves/minute. However, it appears that the frequency of slow waves within a region is to some extent species- and location-specific as mice may have as many as 30 waves/minute in this region (Koh *et al.* 1998).

The slow wave pattern in the colon appears to be much more complex than that of the small intestine as noted in several studies (Taylor *et al.* 1975; Duthie and Kirk 1978; Sanders and Smith 1986b; Brookes *et al.* 1987; Rae *et al.* 1998; Plujà *et al.* 2001). One study in the human colon demonstrated the "regular" slow wave pattern originating at the submucosal aspect of the circular muscle with a frequency of 2-4 contractions/minute (Rae *et al.* 1998). However, near the myenteric border of the circular muscle layer, a rapid fluctuation of the resting membrane potential with a mean frequency of 18 contractions/minute was recorded (Rae *et al.* 1998). This corresponds to the previously described findings in the canine colon where this activity was termed myenteric potential oscillations (MPOs) (Smith *et al.* 1987b). In both studies it was also noted that slow waves and MPOs summated in the central region of the circular muscle layer producing a complex pattern of activity that regulates contractile amplitude and frequency (Smith *et al.* 1987b; Rae *et al.* 1998). In other words, three types of patterns were observed within the same colonic region. It was proposed that the MPOs in the myenteric plexus region arise from a second pacemaker site in this region (Durdle *et al.* 1983; Smith *et al.* 1987b; Rae *et al.* 1998; Plujà *et al.* 2001). Slow wave patterns in the equine colon have not yet been established.

1.4.4 Involvement of ICC in intestinal motility disorders

As the importance of ICC in the generation and maintenance of normal intestinal motility patterns became apparent, researchers started to investigate if changes to ICC networks could be involved in human motility disorders. Indeed, reduced or absent ICC

networks were subsequently detected in a number of GI tract motility disorders in human neonates such as meconium impaction (Yoo *et al.* 2002), hypertrophic pyloric stenosis (Langer *et al.* 1995), anorectal malformations (Kenny *et al.* 1998a) and transient neonatal intestinal pseudo-obstruction (Kenny *et al.* 1998b; Yamataka *et al.* 1998) and it was proposed that this may have been involved in the abnormal motility patterns observed. In the human adult, slow transit constipation constitutes a major problem and reduced ICC volume has been implicated in its pathophysiology (He *et al.* 2000; Knowles and Martin 2000). Similarly, a deficiency of c-Kit positive cells has been found in human patients with chronic idiopathic intestinal pseudo-obstruction, a syndrome characterised by symptoms of bowel obstruction in the absence of obstructing lesions (Isozaki *et al.* 1997). Reduced ICC number and ultrastructural changes to ICC morphology in human adult megacolon and ulcerative colitis respectively, have also been reported (Rumessen 1996; Faussone-Pellegrini *et al.* 1999).

Recently, the ICC have also been identified in the horse and their distribution throughout the GI tract characterised in the normal animal (Hudson *et al.* 1999). This formed the basis for a subsequent study that demonstrated reduced ICC densities in horses with EGS (Hudson *et al.* 2001b). EGS is an important cause of mortality and morbidity of unknown aetiology in the equine population in temperate areas of the Northern and Southern hemisphere e.g. Northern Europe and South America (Doxey *et al.* 1991; Uzal *et al.* 1997a, b). The clinical signs observed reflect the underlying pathology involving neuronal degeneration and loss in both the enteric and autonomic nervous systems (Obel 1955; Doxey *et al.* 1992; Pogson *et al.* 1992; Scholes *et al.* 1993). It was proposed that

the reduction in ICC densities in affected animals contributed to the abnormal intestinal motility patterns experienced by these horses (Hudson *et al.* 2001b). A subsequent *in vitro* electrophysiological study demonstrated the presence of slow wave activity in the ileum of horses with EGS, although a significantly lower frequency compared to normal animals was recorded (Hudson *et al.* 2002).

Further investigations are warranted to determine any possible involvement of the ICC in other equine gastrointestinal motility disorders, especially considering the impact intestinal motility disorders represent in the equine population and the large proportion of these that remain undiagnosed.

1.5 Aims of thesis

The main aim of this thesis was to investigate the role of the ICC in health and disease of the equine intestinal tract using immunohistochemical, molecular and electrophysiological techniques. This included:

- An immunohistochemical investigation, targeting the c-Kit receptor, of the normal development of ICC in the equine foetus. As well as obtaining further information on the ICC in the horse, this study was carried out in order to enable interpretation of samples collected from equine neonates with intestinal motility dysfunction in future studies.

- An immunohistochemical investigation comparing ICC densities in horses with and without intestinal motility disorders testing the hypothesis that these are reduced in some diseased animals. Alternative ICC labelling as well as neuronal densities were also evaluated.

- An investigation into the level of c-*kit* gene expression, encoding the receptor tyrosine kinase c-Kit of ICC, in normal versus diseased animals using real-time quantitative polymerase chain reaction techniques. It was proposed that any changes to ICC densities were a reflection of c-*kit* gene expression and hence these results were compared to a parallel immunohistochemical evaluation.

- An investigation into the electrical events of the equine large colon using *in vitro* intracellular recording techniques in order to help expand our knowledge of intestinal motility patterns in this part of the intestinal tract.

2 IMMUNOHISTOCHEMICAL STUDIES

2.1 Introduction and aims

The discovery that ICC could be identified through immunohistochemical labelling techniques by targeting its c-Kit receptors allowed for rapid advances in not only understanding the normal development and function of these cells, but also in demonstrating their involvement in many human neonatal and adult GI motility disorders (Sanders *et al.* 1999; Vanderwinden and Rumessen 1999). This technique was also used by Hudson *et al.* (2001c) who demonstrated a reduction in ICC densities in horses with EGS compared to control animals. Because of the importance of the c-Kit receptor in maintaining ICC function (Torihashi *et al.* 1995, 1999), it was proposed that the observed reduction in c-Kit-immunoreactive ICC was likely to be involved in creating abnormal motility patterns in these patients (Vanderwinden and Rumessen 1999; Hudson *et al.* 2001c).

The current immunohistochemical investigation set out to test the hypothesis that changes to ICC networks and densities are also present in adult horses with intestinal motility disorders other than EGS. A smaller study also evaluated samples collected from horses that had recovered from a previous episode of chronic EGS. The aim of this study was to investigate the possibility that recovered EGS horses have an intact ICC network that maintains normal intestinal motility patterns in spite of a severely disrupted ENS.

Many GI motility disorders in the equine neonate are associated with immaturity or dysmaturity (Palmer 1985; Wilson and Cudd, 1990; Vaala 1994). An important aim of this study was to investigate the normal development and distribution of ICC in the equine foetus and neonate as this has not been previously characterised in the horse. It would also enable future investigations into a possible involvement of ICC in equine neonatal intestinal motility disorders.

Currently, there is no information available on ICC in other members of the Equidae family. As part of this investigation, intestinal samples were collected from an aged population of donkeys that included both healthy and diseased animals. It was proposed that the density and distribution of ICC would mirror that observed in the equine population. Additionally, this study served as a preliminary investigation into what appears to be a seasonal problem in this particular group of donkeys.

Presently, the only generally recognised immunohistochemical method of identifying ICC in the adult GI tract involves targeting the c-Kit receptor. The current study evaluated the labelling pattern of the intermediate filament marker vimentin in small and large intestinal samples collected from both normal and diseased adult horses in order to assess its potential as an alternative marker of ICC.

As described in the previous chapter, the normal intestinal motility pattern is a complex process involving not only ICC but also smooth muscle cells and an intact enteric neuronal network. A separate study compared neuronal densities in normal versus

diseased animals using a neuronal marker, protein gene product (PGP 9.5). This marker has been used previously in human clinical studies to assess enteric neuronal densities in conditions such as Hirschprung's disease (Sams *et al.* 1992; Oh *et al.* 2002) and idiopathic megarectum and megacolon (Gattuso *et al.* 1996, 1997). It has not been previously used in the investigation of equine intestinal motility disorders. Two H&E studies demonstrated a significant reduction in myenteric neuronal densities in horses with acute and chronic obstructive disorders of the caecum and large colon (Schusser and White 1997; Schusser *et al.* 2000). It was proposed that these changes may have played a part in the increased risk of recurrent colic from colonic or caecal dysfunction in these horses (Schusser *et al.* 2000). As several horses in the current investigation also had a history of recurrent colic episodes, this hypothesis was tested in this horse population using PGP 9.5 as a neuronal marker.

2.2 Materials and methods

2.2.1 Animals and samples used in the studies

2.2.1.1 Ontogeny of ICC

Surplus intestinal tissue samples from 22 naturally-aborted equine foetuses were collected during routine abortion investigations. Two foetuses were examined as part of a *post mortem* investigation associated with the death of the mares. The ages of the foetuses ranged from 6-11 months of gestation. Two foetuses included in this study were

aborted from pony mares, while the remaining animals were all Thoroughbreds. As there was sometimes a delay before the foetus was presented for *post mortem* examination, 10 of the 22 foetuses had to be excluded from the study due to the degree of tissue autolysis (see section *2.2.2.1*). In addition, surplus intestinal tissue samples were collected following the routine *post mortem* investigations of 2 euthanised Thoroughbred foals aged 4 and 8 weeks respectively. The causes of abortion or euthanasia of all animals included in this study are listed in Table 1.

Table 1: Causes of abortion and euthanasia in animals included in the ICC ontogeny study.

Animal	Age	Cause of abortion/death
Foetus 1	F6	Mare euthanised due to terminal gastrointestinal disease
Foetus 2	F7	Premature placental separation, no evidence of infectious cause
Foetus 3	F8	Mare euthanised following spinal trauma
Foetus 4	F9	Premature placental separation, no evidence of infectious cause
Foetus 5	F9	Placental insufficiency associated with fungal placentitis
Foetus 6	F9	Focal placentitis and amnionitis, possible infectious cause
Foetus 7	F10	Acute toxic colitis of mare resulting in abortion
Foetus 8	F10	Premature placental separation, cause unknown
Foetus 9	F10	Uterine body pregnancy resulting in hydrops causing chronic amnionic and chorioallantoic thickening and haemorrhage
Foetus 10	F11	Dystocia, hypoxia and cardiovascular collapse. Birth 1 week earlier than estimated for unknown reasons.
Foetus 11	0	Dystocia; died during caesarean section
Foetus 12	0	Premature placental separation and umbilical cord compression during birth
Foal 1	1	Tyzzer disease (Bacillus piliformis)
Foal 2	2	Cardiovascular collapse associated with jejunal volvulus

Key: F6: 6th month of gestation etc. For foal age 1 and 2 represents 1 and 2 months of age respectively.

Intestinal samples were collected from all animals at anatomically-defined sites. These were ileum (at a position level with the midpoint of the ileocaecal fold), caecal base, pelvic flexure apex (located at the junction between the left ventral colon and left dorsal colon), and the distal third of small colon.

All samples apart from one (Foetus 1) were obtained in collaboration with Rossdale and Partners, Newmarket, Suffolk.

2.2.1.2 ICC colic study

Surplus intestinal tissue samples were collected during surgery from 44 horses of mixed breeds undergoing exploratory laparotomy for colic. Intestinal tissue samples were also collected from 11 control horses of mixed breeds immediately (within 1 hour) following euthanasia by intravenous administration of quinalbarbitone sodium BP (400mg/ml)/cinchocaine hydrochloride BP (25mg/ml) (Somulose, Arnolds, Shropshire, UK).

From the colic group, 10 horses had a strangulating obstruction of the small intestine caused by either epiploic foramen entrapment (n=5) or by a pedunculated lipoma (n=5) (Table 2). Samples collected from this group included sections of jejunum or ileum from the non-ischaemic margin of resected small intestinal tissue. Although an attempt was made to select tissue that was closest to or included ileum to match the anatomical site

of the control samples, this was sometimes not possible if the strangulating lesion involved jejunum only (n=5) (Table 2).

The remaining 34 horses had a large colon disorder comprising a primary pelvic flexure impaction (PFI) (n=7) or large colon accident (LCA) involving large colon displacement or torsion (n=27). All samples collected from this group were surplus tissue following a pelvic flexure apex biopsy submitted for histological analysis and collected during an enterotomy procedure prior to large colon evacuation. All PFI, of which 5 were sand impactions, as well as 3 left dorsal colon displacements had been refractory to initial medical treatment before corrective surgery was carried out. In the remaining 24 animals with a LCA, deteriorating clinical parameters and poor response to analgesic administration dictated that initial medical treatment was not considered appropriate and surgery was carried out immediately following arrival to the referral hospital.

Of the 34 animals in the large colon disorder category, 15 had a history of previous colic episodes of which 4 had undergone previous surgery to correct a condition identical to the current one (1 PFI, 1 recurrent colic without an obvious lesion at surgery and 2 large colon displacements). Three cases in the large colon group were excluded from the study based on the degree of tissue degradation (see section *2.2.2.1*) taking the total number of horses included in this group to 31 (Table 2).

The ages of horses included in the colic group ranged from 1-28 years (median 12.0 years, mean 11.9 years) and consisted of 21 geldings, 19 mares and 1 stallion. Of the 10

horses with a small intestinal lesion, the age ranged from 7-28 years (median 16.5 years, mean 13.4 years), and comprised 1 stallion, 6 geldings and 3 mares. The remaining 31 horses, all with a large colon disorder, had an age range 1-24 years (median 12.0 years, mean 11.5 years) and consisted of 15 geldings and 16 mares.

From each control animal, sections of ileum and pelvic flexure apex were collected at anatomical sites identical to that described in the foetal developmental study. All animals in the control group were euthanised due to conditions not involving the GI tract, of which the majority involved orthopaedic disease. In addition, one horse was euthanised due to a cardiac disorder and one broodmare was put down due to a chronic reproductive problem. None of the control animals had a known history of previous colic episodes. The ages of this group of horses ranged from 1-33 years (median 12.0 years, mean 13.4 years) and included 1 stallion, 7 geldings and 3 mares.

Table 2A: Details on control horses included in the ICC colic study.

Horse	Age (Yrs)	Sex	Diagnosis	Sample collected	Previous colic episodes
Control horses					
01/573	4	G	Lame	Il/ PF	No
01/176	12	G	Lame	Il/ PF	No
99/1024	16	F	Lame	Il/ PF	No
01/488	1	M	Lame	Il/ PF	No
01/462	8	G	Lame	Il/ PF	No
02/252	33	G	Lame	Il/ PF	No
00/364	12	F	Lame	Il/ PF	No
02/56	6	G	Lame	Il/ PF	No
01/805	7	G	Lame	Il/ PF	No
SSPCA	31	G	Cardiac	Il/ PF	No
03/381	18	F	Reproductive	Il/ PF	No

Key: F: Female; G: Gelding; M: Male (entire); Il: Ileum; Jej: jejunum: PF: Pelvic flexure.

Table 2B: Details on colic horses included in the ICC colic study.

Horse	Age (Yrs)	Sex	Diagnosis	Sample collected	Previous colic episodes
SI colic					
25995	18	F	EFE	Il	No
24995	15	F	EFE	Jej	No
25263	9	M	EFE	Jej	No
02/97	7	G	EFE	Il	No
02/526	8	G	EFE	Il	No
25113	15	F	PL	Il	No
25105	28	G	PL	Jej	No
25322	20	G	PL	Jej	No
25932	24	G	PL	Il	No
25849	23	G	PL	Jej	No
LI colic					
25532	14	F	LCT	PF	No
25698	12	G	LLCD	PF	No
25382	11	F	PFI (SI)	PF	Yes
26046	7	F	Unknown	PF	Yes + Surgery
25747	17	F	LCT	PF	No
25999	17	F	RLCD	PF	No
26136	5	F	PFI	PF	Yes
25642	15	F	RLCD	PF	No
26818	1	F	LCT	PF	No
26944	12	F	LLCD	PF	No
26434	19	F	LCT	PF	Yes
26617	8	G	RLCD	PF	Yes
26631	15	G	LCT	PF	Yes
25182	14	G	RLCD	PF	Yes
25022	3	F	PFI (SI)	PF	Yes + Surgery
25154	18	F	PFI (SI)	PF	No
26133	17	G	PFI (SI)	PF	Yes
25597	11	F	LCT	PF	No
25045	24	G	PFI (SI)	PF	Yes
25132	14	G	RLCD	PF	Yes
26697	10	G	RLCD	PF	No
26681	11	G	LLCD	PF	No
27325	17	F	LLCD	PF	No
26283	12	G	LCT	PF	No
26597	12	G	RLCD	PF	Yes
26383	12	F	LCT	PF	No
26852	4	G	RLCD	PF	No
02/784	2	G	RLCD	PF	No
01/242	6	G	RLCD	PF	Yes + Surgery
02/302	5	G	RLCD	PF	Yes + Surgery
26885	10	F	LCT	PF	Yes

Key: SI: Small intestine; LI: Large intestine; F: Female; G: Gelding; M: Male (entire); Il: Ileum; Jej: jejunum: PF: Pelvic flexure; EFE: Epiploic foramen entrapment; PL: Pedunculated lipoma; PFI: pelvic flexure impaction; PFI (SI): pelvic flexure impaction (sand impaction); LCT: Large colon torsion; LLCD: Left large colon displacement; RLCD: Right large colon displacement; Unknown: Diagnosis unknown.

The majority (n=36) of clinical samples included in this study were collected in collaboration with the University of Liverpool, Leahurst, Liverpool, while the remaining samples (n=5) as well as all control animals (n=11) were obtained from The Royal (Dick) School of Veterinary Science (R(D)SVS), Easter Bush, Edinburgh. All samples were obtained with the owners' written consent.

2.2.1.3 ICC in recovered chronic EGS cases

Two horses were included in this study, both of which had been medically diagnosed at the Large Animal Hospital of the R(D)SVS. A diagnosis of EGS had been made in both cases on the basis of clinical signs which included weight loss, mild and intermittent colic episodes, muscle fasciculations, ptosis, dysphagia, rhinitis sicca and anorexia. In the second horse, a positive result on a phenylephrine eye drop test (Hahn and Mayhew 2000) had also been obtained.

The first horse was a 16-year-old Thoroughbred-cross gelding that had been diagnosed with chronic EGS, nursed and subsequently discharged from the Large Animal Hospital 11 years previously. Since then, the horse had not displayed any residual clinical signs of intestinal dysfunction. A chronic orthopaedic problem subsequently warranted euthanasia and the horse was readmitted to the Large Animal Hospital for a *post mortem* examination.

The second horse was an 8 year-old-Cob mare that had also been successfully nursed at the Large Animal Hospital following a medical diagnosis of chronic grass sickness. The mare was discharged 6 weeks following Hospital admission. After making an initial good progress at home with further weight gain, the mare subsequently started to experience repeated episodes of oesophageal obstruction (choke) and was readmitted to the Large Animal Hospital 7 months following discharge. A diagnosis of megaoesophagus was made and the mare was subsequently euthanised due to the poor prognosis.

Following euthanasia, a section of ileum as well as cranial cervical and cranial mesenteric ganglia were collected from both horses. In addition, a section of jejunum was also collected from the first horse. All samples were collected with the written consent of the owners.

2.2.1.4 ICC in the donkey

Surplus intestinal tissue samples were collected from 12 donkeys following routine *post mortem* examinations. In 7 animals, euthanasia was related to clinical signs indicating an abdominal problem, while in the remaining 5 other conditions were diagnosed as described in Table 3. As seen from this table, one of these animals (Donkey 6) was subsequently diagnosed with a chronic liver problem and was hence included in the control group rather than in the "colic" group. All animals with a suspected abdominal problem had been given some form of empirical medical treatments such as analgesic

administration. However, as these failed to resolve the problem, the decision was taken to euthanise the individual animals rather than pursue further treatments including exploratory abdominal surgery. The latter was not considered to be in the animals' best interest primarily due to their advanced age and general physical condition, making them poor surgical candidates.

In all animals, apart from 1 (Donkey 12), sections of ileum and pelvic flexure were collected at anatomically defined sites as described above in the ICC foetal developmental study. In 1 individual with an intestinal disorder (Donkey 12), a section of pelvic flexure only was collected. As no significant degree of tissue degeneration (see section *2.2.2.1*) was present in any of the samples collected, all were included in the study.

All donkeys were aged with a range of 26-35 years (median 28.0 years, mean 29.3 years), and consisted of 1 stallion, 7 geldings and 4 mares. The age range of the control group was 26-32 years (median 27.0 years, mean 28.1 years) and comprised 2 geldings and 4 mares. The age range for the colic group was 27-35 years (median 30.0 years, mean 30.3 years) and comprised 1 stallion and 5 geldings.

The condition of the teeth was also assessed in each animal. These were graded as good, moderate or poor depending on the number of teeth missing, the quality and extent of functional occlusal surfaces and any evidence of gingival disease. Based on this

classification, only 1 animal had good teeth and healthy gingiva, 3 were moderate while the remaining 8 had poor dentition (Table 3).

All animals at the time of death were housed in a loose barn system with straw bedding and fed primarily hay and straw.

All samples were collected in collaboration and permission with the Donkey Sanctuary, Sidmouth, Devon which also owned all animals included in the study.

Table 3: Details of donkeys included in the immunohistochemical study.

Animal	Age (Yrs)	Sex	Reason for euthanasia	Diagnosis	Teeth
Controls					
Donkey 1	32	F	Lame	Laminitis	Moderate
Donkey 2	26	G	Neurological	Unknown	Moderate
Donkey 3	31	G	Dental	Dental	Poor
Donkey 4	28	F	Renal	Nephrosis	Poor
Donkey 5	26	F	Respiratory	Pneumonia	Moderate
Donkey 6	26	F	Liver	CLF	Poor
LI colic					
Donkey 7	27	G	Weight loss	PFI	Poor
Donkey 8	35	G	Colic	PFI	Poor
Donkey 9	33	M	Suspected liver disease	PFI	Poor
Donkey 10	32	G	Weight loss	PFI	Poor
Donkey 11	28	G	Distended abdomen	PFI	Poor
Donkey 12	27	G	Distended abdomen	PFI	Good

Key: F: Female; G: Gelding; M: Male (entire); PFI: pelvic flexure impaction; LI colic: large intestine colic; CLF: Chronic liver fibrosis.

2.2.1.5 ICC labelling: Vimentin

Intestinal tissue samples were collected immediately (within 1 hour) following euthanasia from 7 control horses. All animals had been euthanised for conditions not relating to the GI tract and had no known previous history of colic. In 3 of these animals both ileum and pelvic flexure were collected, while in the remaining 4 only ileum (n=3) or pelvic flexure were collected (n=1). The age range of the horses included in this group was 3-33 years (median 12.0 years, mean 13.7 years) and consisted of 4 geldings and 3 mares.

In addition, intestinal samples were collected from 8 horses undergoing exploratory laparotomy for colic. Of these animals 3 horses had an obstructive lesion of the small intestine while the remaining 5 had a large colon obstructive disorder. The samples collected from this group included sections of jejunum (n=1) or ileum (n=2) from the non-ischaemic margin of resected small intestinal tissue (n=3) or a section of surplus pelvic flexure biopsy material (n=5) collected during an enterotomy procedure (n=5). The age range of this group was 5-17 years (median 10.5 years, mean 10.2 years) and consisted of 4 geldings and 4 mares. All animals included in this study are described in Table 4. All samples were collected with the owners' written consent.

Table 4: Details on horses included in ICC vimentin study.

Horses	Age (Yrs)	Sex	Diagnosis	Sample collected
Control group				
01/573	4	G	Lame	Ileum/ Pelvic flexure
03/381	18	F	Reproductive disease	Ileum/ Pelvic flexure
01/765	15	F	Respiratory disease	Ileum/ Pelvic flexure
01/462	11	G	Lame	Ileum
00/364	12	F	Lame	Ileum
02/252	33	G	Lame	Ileum
03/303	3	G	Lame	Pelvic flexure
Colic group				
02/90	12	G	Epiploic foramen entrapment	Ileum
03/435	12	G	Eosinophilic enteritis	Ileum
00/656	8	F	Mesenteric rent	Jejunum
25747	17	F	LCT	Pelvic flexure
28287	14	F	LCT	Pelvic flexure
28225	5	F	LLCD	Pelvic flexure
27644	5	G	LLCD	Pelvic flexure
02/97	9	G	RLCD	Pelvic flexure

Key: G: gelding; F: female; LCT: large colon torsion; LLCD: left large colon displacement; RLCD: right large colon displacement.

2.2.1.6 Neuronal immunohistochemical labelling-PGP 9.5

Intestinal samples from 17 control horses were collected immediately (within 1 hour) following euthanasia for conditions not relating to the GI tract. The ages of the animals in this group ranged from 1-33 years (median 11.0 years, mean 12.3 years) and comprised 1 entire male, 11 geldings and 5 mares. The samples collected included a section of ileum and/or pelvic flexure as described. As it was not always possible to obtain both ileal and pelvic flexure samples from all horses included in this study, a total of 12 ileal and 8 pelvic flexure control samples were collected (Table 5). The small intestinal control group ranged from 1-33 years (median 11.5 years, mean 13.5 years) and comprised 1 entire male, 8 geldings and 3 mares, while the large intestinal control

Table 5: Details on horses included in PGP 9.5 study.

Horse	Age (Yrs)	Sex	Diagnosis	Sample collected	Previous colic episodes
Control group					
01/573	4	G	Lame	Il/ PF	No
03/488	11	G	Lame	Il/ PF	No
01/488	1	M	Lame	Il/ PF	No
01/176	12	G	Lame	Il	No
99/1024	16	F	Lame	Il	No
01/462	11	G	Lame	Il	No
02/252	33	G	Lame	Il	No
03/381	18	F	Reproductive	Il	No
00/364	12	F	Lame	Il	No
02/56	6	G	Lame	Il	No
01/805	7	G	Lame	Il	No
SSPCA	31	G	Cardiac	Il	No
99/788	19	G	Lame	PF	No
03/303	3	G	Lame	PF	No
01/765	15	F	Lame	PF	No
03/124	9	G	Lame	PF	No
Wobbler	2	F	Central neuro.	PF	No
SI Colic					
02/97	7	G	EFE	Il	No
02/90	12	G	EFE	Il	No
27870	24	F	Lipoma	Jej	No
02/209	14	G	Meckel's Div	Jej	No
00/656	8	F	Mes. Rent	Jej	No
03/435	12	G	Eosinophil	Il	No
9/6/03	19	G	Lipoma	Il	No
02/110	14	G	EFE	Il	No
26271	24	F	Lipoma	Jej	No
LI Colic					
01/242	6	G	RLCD	PF	Yes + Surgery
02/302	5	G	RLCD	PF	Yes + Surgery
25022	3	F	PFI (SI)	PF	Yes + Surgery
25045	24	G	PFI (SI)	PF	Yes
25382	11	F	PFI (SI)	PF	Yes
25532	14	F	LCT	PF	No
25597	11	F	LCT	PF	No
25747	17	F	LCT	PF	No
26383	12	G	LCT	PF	No
26852	4	G	RLCD	PF	No
28287	14	F	LCT	PF	Yes + Surgery
27644	5	G	LLCD	PF	No
27870	11	F	RLCD	PF	No
Red	11	G	RLCD	PF	No
28225	5	F	LLCD	PF	Yes
Annie	9	F	LLCD	PF	No
27670	9	G	LCT	PF	No
28483	2	F	LLCD	PF	No
28570	3	F	LLCD	PF	No

Key: F: Female; G: Gelding; M: Male (entire); Il: Ileum; Jej: jejunum: PF: Pelvic flexure; EFE: Epiploic foramen entrapment; Lipoma: Pedunculated lipoma; Meckels' Div: Meckel's diverticulum; Mes. Rent: Mesenteric rent; Eosinophil.: Focal strangulating eosinophilic lesion; Central neuro: Central neurological problem; PFI (SI): pelvic flexure impaction (sand impaction); LCT: Large colon torsion; LLCD: Left large colon displacement; RLCD: Right large colon displacement.

group comprised 1 entire male, 5 geldings and 2 mares with an age range of 1-19 years (median 6.5 years, mean 8.0 years).

In addition, intestinal tissue samples were collected from 28 horses undergoing exploratory laparotomy for colic. The age range of this group was 2-24 years (median 11.0 years, mean 11.0 years) and comprised 14 geldings and 14 mares. Small intestinal tissue sections were obtained from the non-ischaemic margin of resected small intestinal tissue from 9 horses with an obstructive lesion of this anatomical region. The samples collected included jejunum (n=4) or ileum (n=5). As for the ICC study, an effort was made to collect samples that were similar or as close to the anatomical site of the control animals as possible. The age range of this group was 7-24 years (median 14.0 years, mean 14.8 years) and consisted of 6 geldings and 3 mares. In addition, surplus tissue samples from pelvic flexure biopsies collected from 19 horses with a large colon obstructive disorder were evaluated. The age range of this group was 2-24 years (median 9.0 years, mean 9.3 years) and consisted of 8 geldings and 11 mares.

The animals in this study included horses from both the equine colic ICC study as well as animals included in the molecular study described in the following chapter of this

thesis. The reason for including horses from both these studies was to obtain a higher collective sample number as there was not always enough tissue available to be used in all immunohistochemical studies. This was particularly true for the large colon colic samples which were all surplus material following a pelvic flexure apex biopsy. The majority of colic samples were collected through collaborative work with the University of Liverpool (n=18), while all control (n=17) and 10 colic samples were collected at the R(D)SVS. All samples were collected with the written consent of the owners.

PGP 9.5 density was also evaluated in the tissue samples collected from the 2 recovered chronic EGS horses as well as in the donkeys described earlier (apart from ileum in Donkey 9 and pelvic flexure in Donkey 4).

2.2.2 Immunohistochemical labelling

Although the above studies investigating c-Kit immunoreactivity were carried out separately, they all followed the same immunohistochemical labelling protocol. This has previously described by Hudson *et al.* (1999, 2001c) and is outlined in detail below. The protocol for vimentin and PGP 9.5 labelling is also described.

2.2.2.1 c-Kit

Following collection, all samples were immediately placed in 10% phosphate-buffered formalin and fixed for at least 24 hours. Tissue processing consisted of rinsing the tissue

samples in running tap water for 1 hour prior to placing them in graded sucrose solutions (10 and 30% sucrose in phosphate-buffered saline), in order to cryo-protect the samples. The samples were frozen rapidly in isopentane (BDH Laboratory Supplies, Poole, UK) pre-cooled in liquid nitrogen or dry ice and subsequently cut at thicknesses of 10 μm and 20 μm. All sections were mounted on Tespa–coated (3-aminopropyltriethoxysilane) (Sigma Aldrich, Poole, UK) slides and allowed to air-dry overnight. After washing the sections in phosphate-buffered saline (PBS, Appendix 1), they were incubated for 30 minutes in 0.3% hydrogen peroxide in methanol in order to quench endogenous peroxidase activity. The sections were then incubated for 1 hour in 1% goat serum (Vector Laboratories, Burlingame, CA, USA) in order to block non-specific antibody binding. After subsequent washes with PBS, the sections were incubated overnight at 4°C in humid chambers in a rabbit-raised polyclonal antiserum to c-Kit (Ab-1, Oncogene Research Products, Cambridge, MA, USA) at a concentration of 1μg. ml^{-1}. The tissue sections were then washed with PBS prior to 1 hour incubation with biotin-conjugated goat anti-rabbit immunoglobulin (Vector Laboratories) at a dilution of 1:200. Immunoreactivity was revealed using the avidin-biotin method (ABC) (Vectastain Elite ABC Kit, Vector Laboratories) using a diaminobenzidine substrate (DAB) (BDH Laboratory Supplies). Sections were dehydrated in ethanol, cleared in xylene and then mounted in Depex (Merck, Glasgow, UK).

For all batches processed, both negative and positive controls were included. In negative controls, the primary antibody was replaced with normal rabbit serum. A tissue sample

from a normal control animal from a previous study that had been shown to have an abundance of c-Kit-immunoreactive ICC was used as a positive control.

ICC were differentiated from round c-Kit-immunoreactive mast cells in the submucosa by presence of cellular processes in ICC, or by staining parallel sections with toluidine blue to demonstrate mast cell metachromasia (Galli *et al.* 1993; Hudson *et al.* 1999).

In addition to immunohistochemical labelling, all processed tissues were stained with haematoxylin and eosin (H&E) in order to assess tissue integrity. This was carried out to help clarify whether reduced or absent c-Kit immunoreactivity was a primary finding or secondary to a degree of tissue degradation. Only samples with good tissue integrity (and not worse than a mild degree of mucosal degradation) were included in order to ensure that immunohistochemical grading was reliable.

2.2.2.2 Vimentin

The processing of tissue samples labelled with this antibody was identical to that of c-Kit with the following exceptions. As vimentin (Sigma Aldrich) is a goat-raised polyclonal antibody, the tissue sections were incubated for 1 hour in 1% rabbit serum (Vector Laboratories) in order to block non-specific antibody binding. Different dilutions (1:10, 1:20 and 1:50) of vimentin were tested on sections from control animals with the optimal results being obtained at 1:50 dilution. This was the dilution used for the subsequent sections. As for c-Kit, tissue sections were incubated overnight in humid

chambers with the primary antiserum before applying the secondary antibody, in this case a biotinylated rabbit anti-goat antiserum (Vector Laboratories) at a dilution of 1:50. The remaining tissue processing was identical to that described for c-Kit.

2.2.2.3 Protein Gene Product (PGP 9.5)

Protein Gene Product (PGP 9.5) is a rabbit-raised polyclonal antiserum (UltraClone Ltd., Isle of Wight, UK). The processing of tissue samples labelled with this antibody was identical to that of c-Kit although the dilution of the primary antibody, PGP 9.5, was 1:8000.

2.2.3 Sample assessment and statistical analysis

2.2.3.1 Ontogeny ICC colic

The results of this study were descriptive only and hence no statistical analysis was performed.

2.2.3.2 ICC colic study

Two independent observers assessed and graded all tissue sections. The grading of each animal was based on the scrutiny of 6 adjacent 10μm sections of tissue. This semi-quantitative grading system has been previously described (Hagger *et al.* 1998a, b, 2000;

Hudson *et al.* 2001c). In the study by Hudson *et al.* (2001c), the absence of c-Kit immunoreactivity was graded 0, sparse 1, moderate 2 and abundant 3. This grading scale was also adopted for the current study. Separate evaluation of the myenteric and circular muscle regions as well as combined c-Kit immunoreactivity was carried out for all samples. In the event of disagreement of grading between the two observers, a consensus grade was assigned after a joint review of the sections. As the grades given were not normally distributed, a Mann-Whitney test assuming a significance level of 5% was used.

Inter-observer variability was assessed using a Mann-Whitney test, again assuming a significance level of 5%.

2.2.3.3 ICC in recovered chronic EGS cases

Although the assessment of the density and distribution of c-Kit-immunoreactive ICC were principally identical to that of the ICC colic study, the small sample size precluded statistical assessment. The findings in this study were therefore descriptive only. H&E evaluation of tissue sections as well as of cranial cervical and cranial mesenteric ganglia was performed by Drs EM Milne and I Begara-McGorum, Veterinary Clinical Pathology Unit, University of Edinburgh, Easter Bush, UK.

2.2.3.4 ICC in the donkey

The assessment and tissue grading of these samples were identical to that of the ICC colic study. The results were analysed using a Mann-Whitney test assuming a significance level of 5%. A possible involvement of dental disease on the incidence of pelvic flexure impaction was additionally evaluated in this study using a Qui-squared test with odds ratio assuming a significance level of 5%.

2.2.3.5 ICC labelling: Vimentin

As for the ontogeny study, this was a descriptive study and therefore no statistical analysis was performed.

2.2.3.6 Neuronal immunohistochemical labelling –PGP 9.5

All samples were evaluated blindly twice, one week apart by one observer similar to that described by Prince *et al.* (2003). A semi-quantitative grading system as described for the ICC colic study was adopted. The grades given were based on the scrutiny of 4 10μm tissue sections from each animal. Immunoreactivity of the myenteric plexus region and circular muscle was evaluated separately. Where mucosa was included in the sections, immunoreactivity of this region was additionally evaluated. It was not always possible to obtain complete full thickness samples in all animals for this study depending on the size and thickness of the original tissue section.

A Mann-Whitney test assuming a significance level of 5% was used when analysing the data. Variability between the grades given on the two separate occasions was also assessed using the same statistical method.

2.3 Results

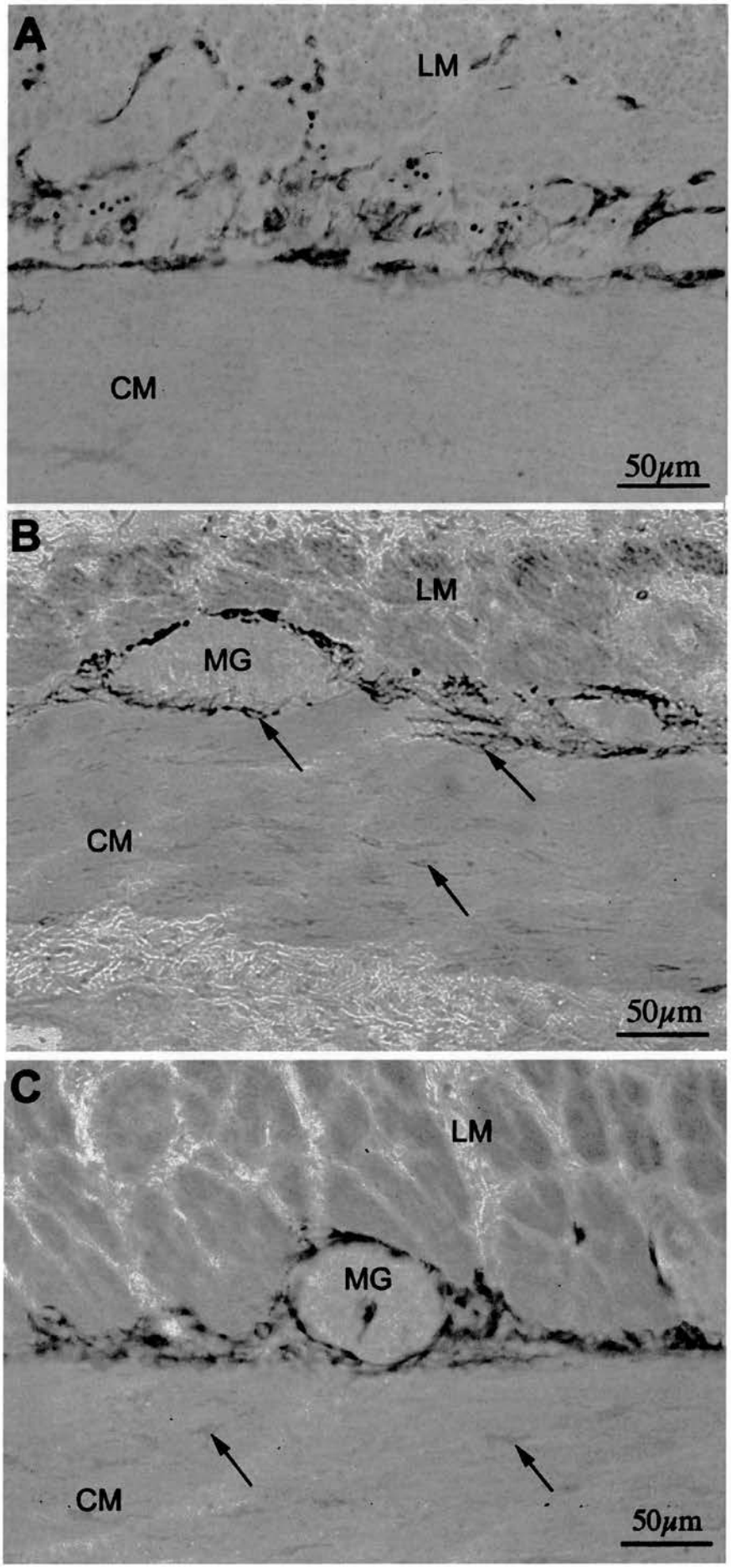
2.3.1.1 Ontogeny of ICC

In the small intestine (ileum) of all the foetuses examined, c-Kit-immunoreactive ICC were identified in the myenteric plexus region displaying a mixture of bipolar and stellate-shaped morphologies. Individual ICC could be identified in the youngest foetuses but in contrast, in the older foetuses and foals, the increasingly dense ICC network that developed surrounding the myenteric ganglia made it more difficult to identify individual cells (Figures 3 and 4). There was a limited and inconsistent degree of branching of ICC from the myenteric plexus region into the longitudinal muscle layer in the foetuses. However, this branching appeared more prominent and consistent in the two neonatal foals examined (Figure 4).

Interstitial cells were also observed in the small intestinal circular muscular layer (Figure 3) in all samples examined. In the youngest foetus (6 months of gestation), few cells were observed within this muscle layer compared to the older foetus (Figure 3) and neonate. The majority of ICC in the circular muscle of the ileum were bipolar in shape,

although in the older foetus there appeared to be a slight increase in the proportion of stellate-shaped cells. The orientation of the long axis of ICC was parallel to the circular muscle fibres.

Figure 3: c-Kit immunoreactivity in the ileum of the small intestine in three foetuses aged 6 (A), 7 (B) and 10 months (C) respectively. Individual c-Kit-immunoreactive ICC can be observed in the myenteric plexus region as well as in the circular muscular layer (arrows). LM: longitudinal muscle layer; CM: circular muscle layer; MG: myenteric ganglia.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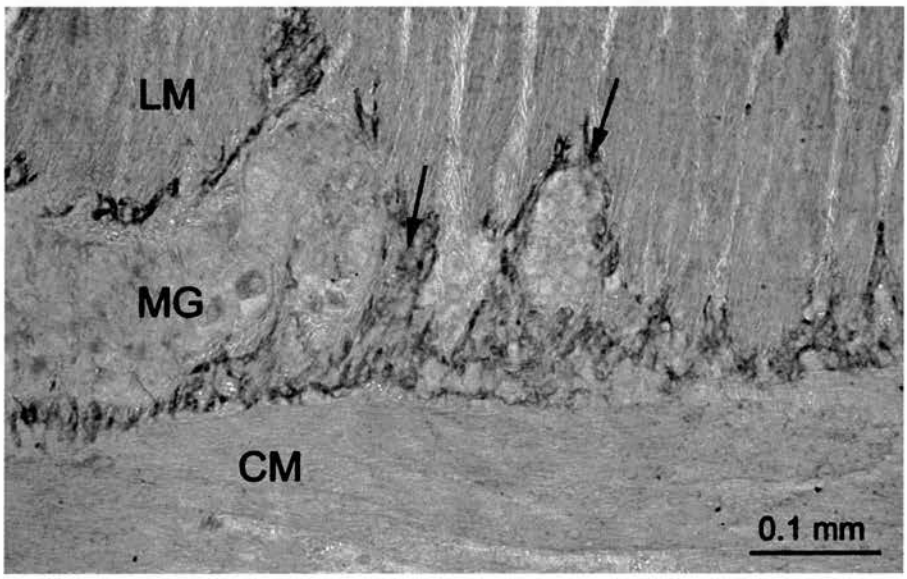
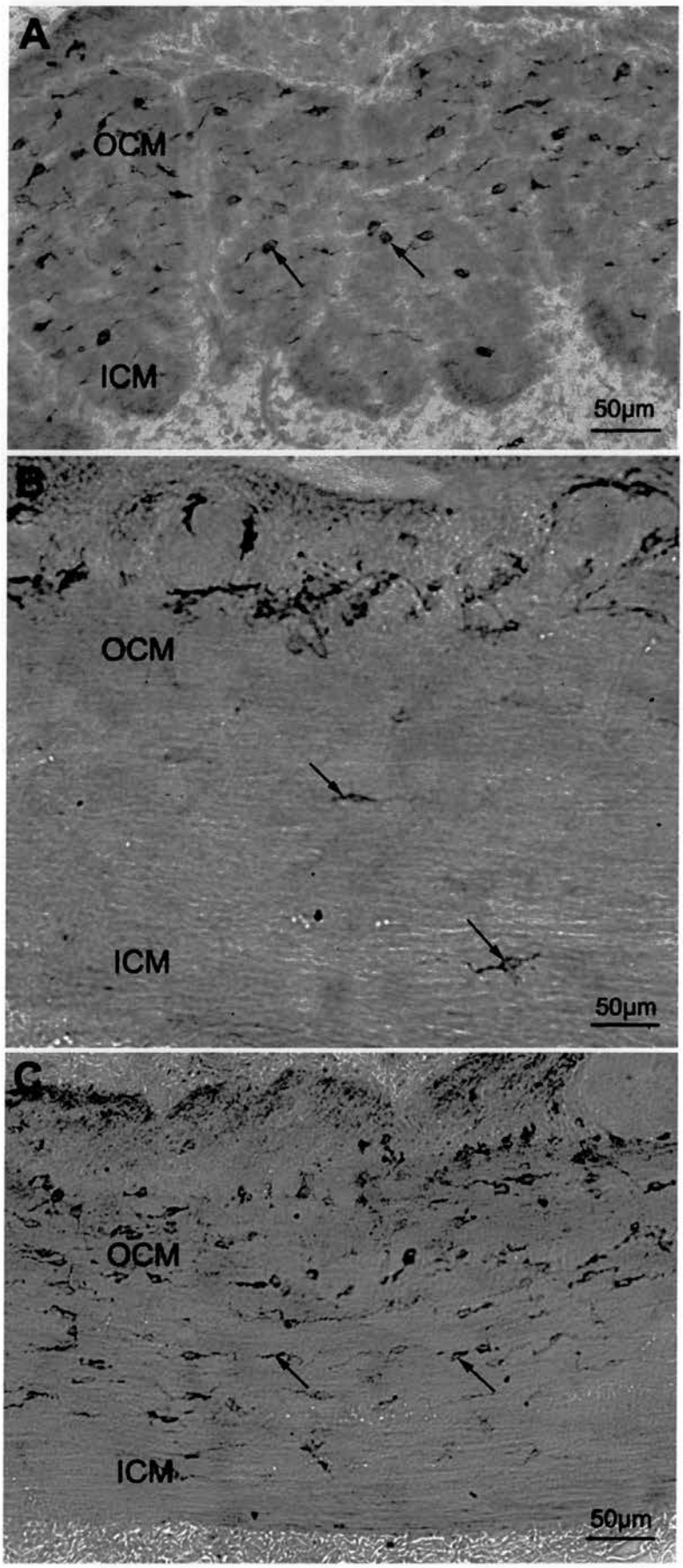


Figure 4: c-Kit-immunoreactivity in the ileum of the small intestine in a 1 month old foal. A dense network of ICC surrounds the myenteric ganglia with branching of ICC from the myenteric plexus region into the longitudinal muscle layer (arrows). LM: longitudinal muscle layer; CM: circular muscle layer.

In the large intestine (caecum, pelvic flexure and small colon), both bipolar and stellate-shaped c-Kit-immunoreactive ICC were present in the myenteric plexus region. It was not possible to determine if one cell-type was more predominant than the other. The ICC did not form the dense networks observed in the small intestine but rather having a more lace-like appearance. The distribution and density of ICC in this region did not appear to change significantly during foetal development or indeed into the neonatal period.

Bipolar and stellate-shaped ICC were also observed in the circular muscle layer of the *muscularis externa* of the large intestine in all samples. However, in contrast to the ileum, there was no clear predominance of either morphological type. A proximal to distal gradient of ICC distribution was detected which was most evident in the youngest foetuses (6-8 months of gestation) but became less distinct as the foetuses developed (Figure 5). A summary of this proximal to distal gradient of distribution is illustrated in Figure 6.

Figure 5: c-Kit immunoreactivity in the large intestine of the developing foetus. Figure A and B: Section of the caecal base and distal small colon respectively of a 7 month old foetus. A rostro-caudal gradient of distribution is evident with more ICC being present in the cacum compared to the small colon. A transmurally decreasing gradient of ICC distribution in the circular muscle layer was observed with the innermost region having a lower ICC density compared to the outer (serosal) region (Figure A). This gradient is still evident in a late gestation foetus (10 months) in the distal small colon (Figure C).
ICM: inner circular muscle region; OCM: outer circular muscle region.


A
OCM
ICM
50μm
B
OCM
ICM
50μm
C
OCM
ICM
50μm

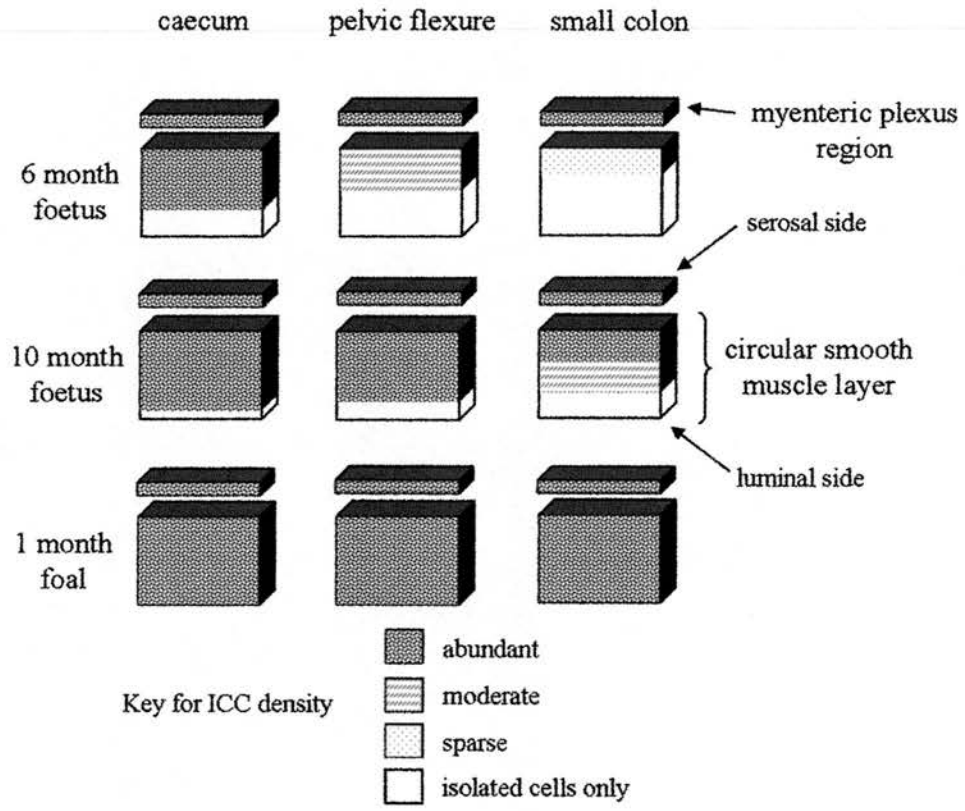


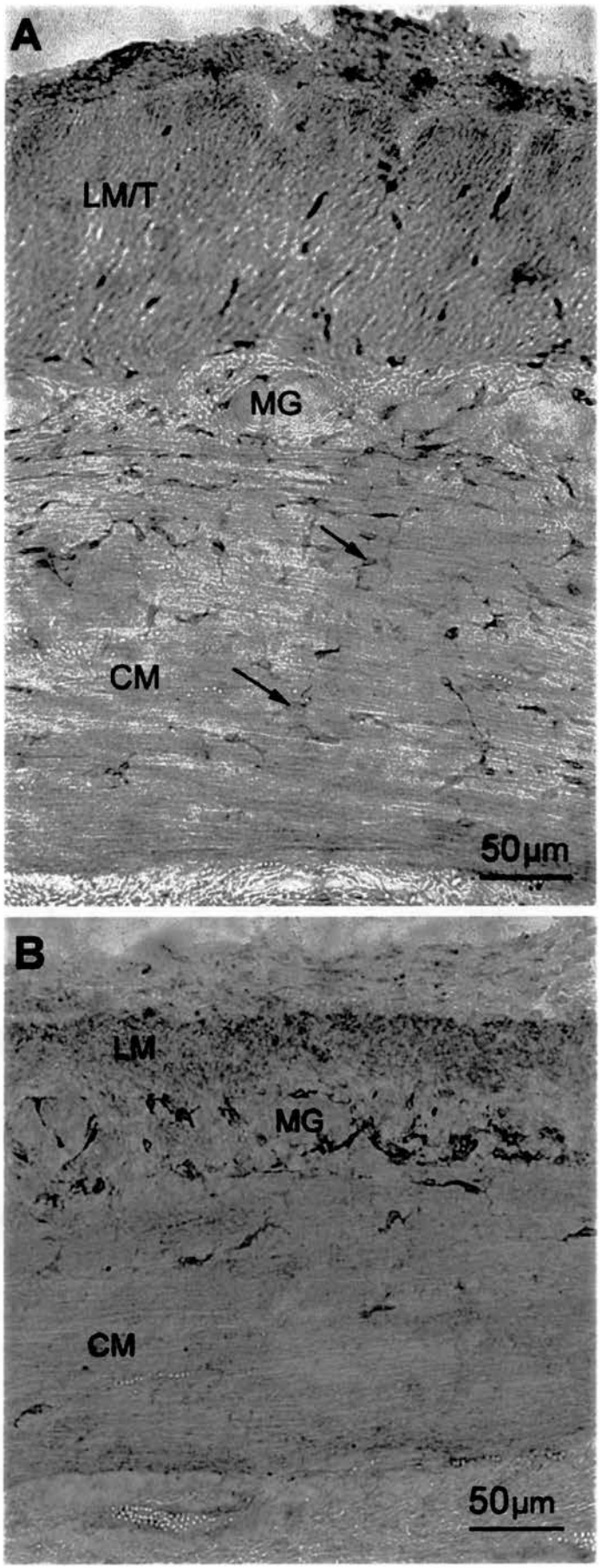
Figure 6: Schematic illustration of the proximal to distal developmental distributions of ICC in the myenteric plexus region and the circular muscle layer of the equine large intestine. The age-related changes in the transmural distribution of ICC within the circular muscle layer are also illustrated.

As well as a proximal-distal gradient of ICC distribution, a transmural gradient was also evident in the circular muscle layer of the large intestine. In the youngest foetuses, the majority of ICC in the most proximal part of the large intestine (caecum) were seen in the outer (more serosal) two thirds of this muscle layer, with the innermost region having a lower ICC density (Figure 5A). However, at the same stage of gestation, in the most caudal part of the large intestine, the distal small colon, very few ICC were observed in any part of the circular muscle layer (Figure 5B). In the developing foetus, the ICC distribution observed in the pelvic flexure (a site approximately midway between the caecum and distal small colon), was less than that of the caecum but more extensive than that of the distal small colon. In late gestation, an area of low ICC density at the innermost part of the circular muscle layer was still evident in the most caudal part of the large intestine (Figure 5C). The transmural gradient of ICC distribution was no longer apparent in the two neonatal animals examined. A schematic representation of this gradient is shown in Figure 6.

It was noted that in the areas where taenial bands were located, ICC density was consistently high compared to areas away from the taenial band (Figure 7). This was true both in the myenteric plexus region and the circular muscle layer, although particularly so for the latter. This was a consistent finding in both the developing foetus as well as the foals irrespective of age and anatomical location.

Figure 7: c-Kit immunoreactivity in the distal small colon of a 7 month old foetus at an area of a taenial band (LM/T) (A) as well as at an area away from it in the same tissue section (B). Numerous ICC can be observed in the circular muscle layer (arrows) in A which contrasts that observed away from the taenial band (B).

LM/T: longitudinal muscle layer/ taenial band; CM: circular muscle layer; MG: myenteric ganglia.


A
LM/T
MG
CM
50μm
B
LM
MG
CM
50μm

2.3.1.2 ICC colic study

Immunohistochemical labelling indicated the presence of c-Kit-immunoreactive ICC in both surgical colic cases as well as control animals. However, the distribution and density varied greatly between cases.

In the small intestine, a continuous, dense band of immunoreactivity could be observed in the myenteric plexus region in the majority of samples from both groups (Figure 8). Because of the density of the c-Kit immunoreactivity, it was difficult to determine the predominant shape of ICC in this region. There was no significant difference in the ICC density grades given in the myenteric plexus region between normal and diseased animals ($p=0.450$). The proportion of grades given in this region in the two groups is illustrated in Figure 9.

As in the myenteric plexus region, there was no significant difference in ICC density between the two groups in the circular muscle layer of the small intestine ($p=0.143$). The proportions of grades given are illustrated in Figure 10. Generally, the density of cells in this region was less than that observed in the myenteric plexus region. However, in some animals, there appeared to be an increased density of ICC at the inner (luminal) third of the circular muscle layer. This was particularly evident in one sample collected from a horse with a small intestinal lesion where a clear interconnecting network of ICC was observed (Figure 11). The majority of cells observed in this particular animal appeared stellate in shape with the orientation of the main cellular processes parallel to that of the

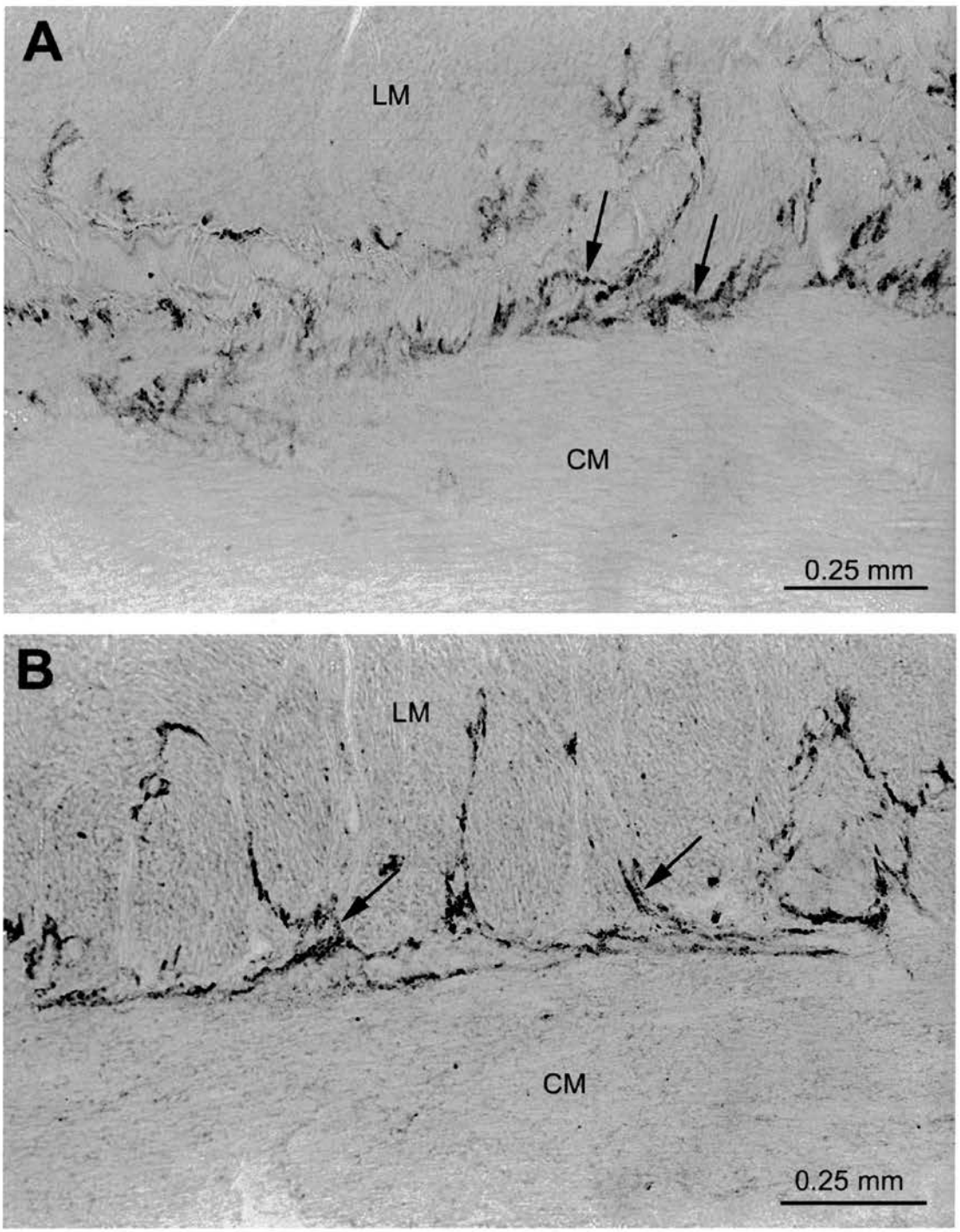


Figure 8: Sections of the myenteric plexus region of the ileum of a control animal (A) and that of a horse with a strangulating lesion of the small intestine (B). There appears to be little difference in the density of c-Kit-immunoreactive ICC between these two samples. Arrows highlight labelled ICC. LM: longitudinal muscle layer; CM: circular muscle layer.

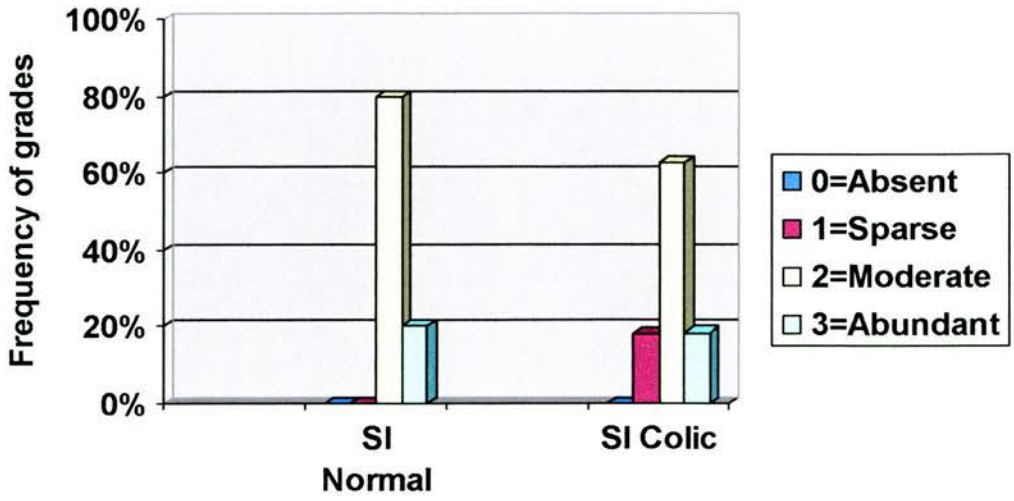


Figure 9: ICC density in the myenteric plexus region in the small intestine of normal horses (n=11) and horses with a small intestinal obstructive lesion (n=10). Frequency grade (%) represents the proportion of specimens that were assigned to a particular density grade: absent (0), sparse (1), moderate (2) and abundant (3).

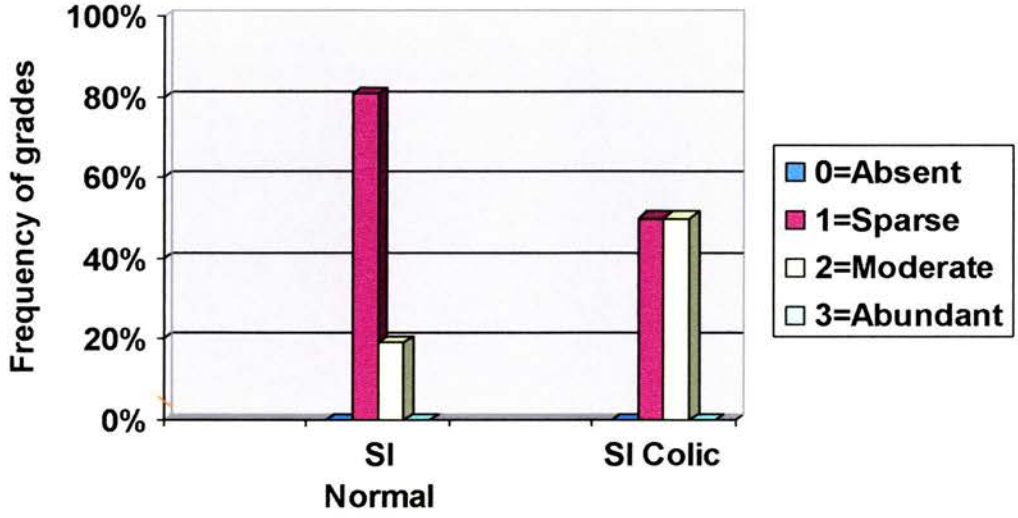


Figure 10: ICC density in the circular muscle region in the small intestine of normal horses (n=11) and horses with a small intestinal obstructive lesion (n=10). Frequency grade (%) represents the proportion of specimens that were assigned to a particular density grade: absent (0), sparse (1), moderate (2) and abundant (3).

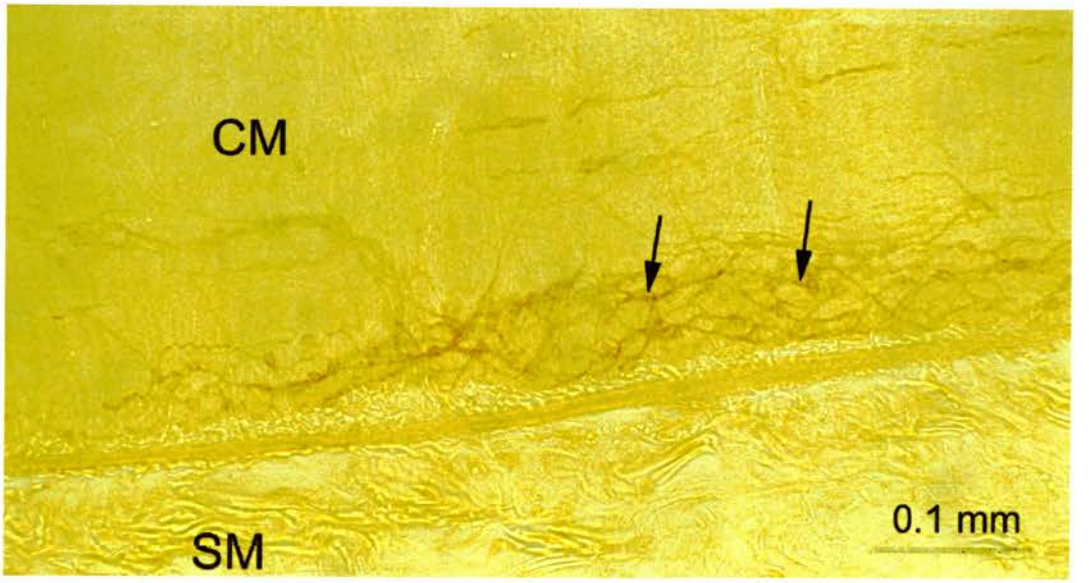


Figure 11: Section of ileum from a horse with an obstructive lesion of the small intestine (epiploic foramen entrapment). A dense network of c-Kit-immunoreactive ICC can be observed along the submucosal border of the circular muscle layer (arrows). CM: circular muscle layer; SM: submucosa.

circular muscle fibres. Throughout the remainder of the circular muscle cell layer, there appeared to be a mixture of uniformly distributed bipolar and stellate-shaped cells.

When the combined grades for the myenteric and circular muscle region were assessed, no significant difference in the density of ICC ($p=0.074$) was evident between the two groups of horses.

Both the density and distribution of c-Kit-immunoreactive ICC in the small intestine differed from that observed in the samples collected from the large colon. In contrast to the former where the immunoreactivity of the myenteric plexus region was very dense making it difficult to identify individual cells, the distribution of cells in the large intestine appeared much more delicate and lace-like. Again it was difficult to get a clear impression of the predominant shape of the ICC observed, although it was possible to make out some individual cells of which both bipolar and stellate shaped cell types were noted (Figure 12). When the ICC density was graded, a significant reduction ($p=0.0004$) in c-Kit-immunoreactive cells in samples collected from diseased animals compared to the control group was evident. The grades allocated to the two groups in the myenteric plexus region are illustrated in Figure 13.

A significant reduction in ICC densities was also evident in the circular muscle layer of the *muscularis externa* in horses with a large colon obstructive disorder compared to the control animals ($p=0.0001$). This difference in grades obtained for the two groups is illustrated in Figure 14. As seen in this bar diagram, little or no c-Kit immunoreactivity

Figure 12: Myenteric ganglia of the pelvic flexure in a normal horse. A more delicate, lacelike distribution of c-Kit-immunoreactive ICC is evident compared to the small intestine. Individual ICC including both bipolar and stellate-shaped ICC are pointed out by the arrows. LM: longitudinal muscle layer.

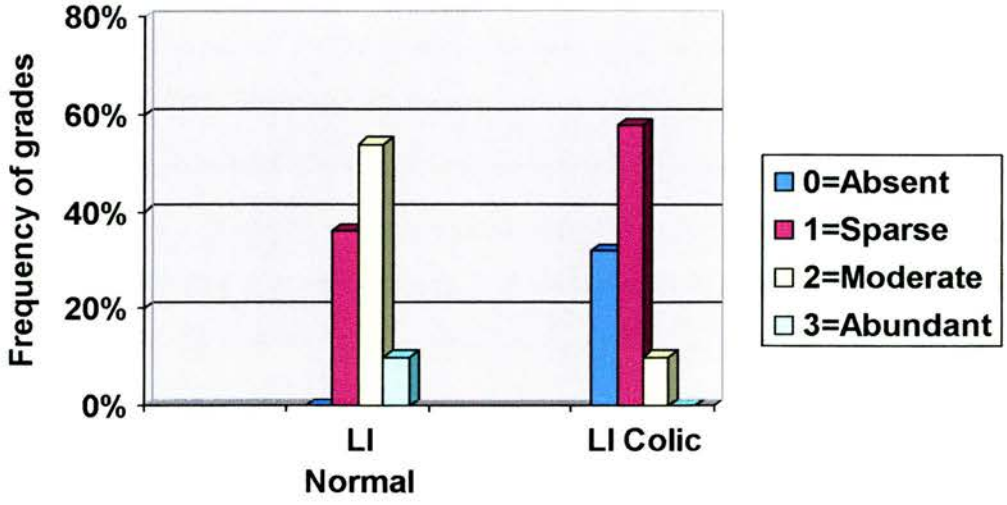


Figure 13: ICC density in the myenteric plexus region in the large intestine of normal horses (n=11) and horses with a large intestinal obstructive lesion (n=31). Frequency grade (%) represents the proportion of specimens that were assigned to a particular density grade: absent (0), sparse (1), moderate (2) and abundant (3).

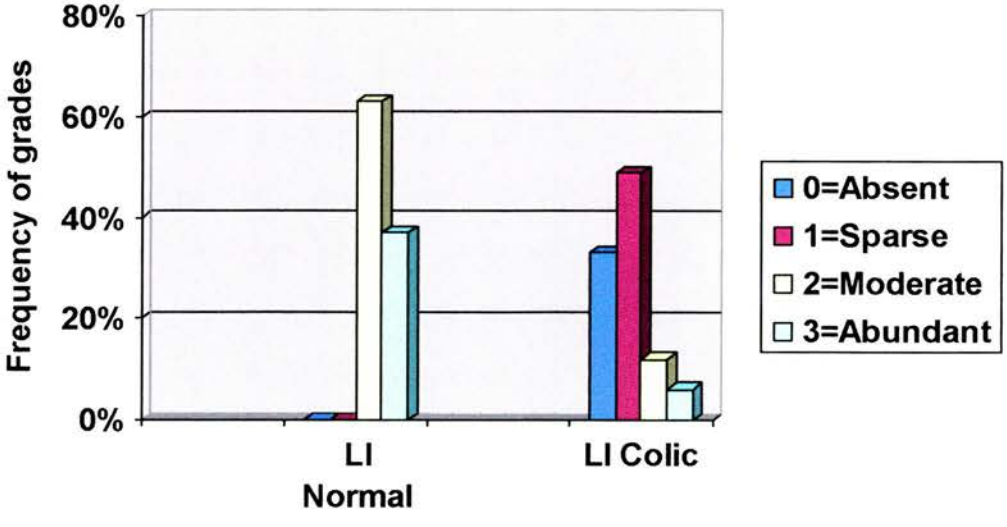


Figure 14: ICC density in the circular muscle region in the large intestine of normal horses (n=11) and horses with a large intestinal obstructive lesion (n=31). Frequency grade (%) represents the proportion of specimens that were assigned to a particular density grade: absent (0), sparse (1), moderate (2) and abundant (3).

was detected in a large proportion of animals in the diseased group. This differed markedly to the majority of control animals which had moderate to abundant c-Kit immunoreactivity. This difference is further illustrated by a representative sample from each group as seen in Figure 15. Where immunoreactivity was detected, in both normal and diseased animals, there appeared to be a mixture of bipolar and stellate-shaped cells with a relatively uniform distribution throughout this muscle layer. As observed in the small intestine, the orientation of the ICC processes was parallel to that of the muscle fibres.

There was a significant reduction in the overall ICC density in horses with a large colon obstructive problem compared to that of the control group ($p=0.0001$).

It was noted that 15 out of the 31 horses with a large colon disorder had a history of previous colic episodes of which 4 animals had undergone corrective abdominal surgery. The diagnosis at the time of surgery for these animals was similar to that of the current episode. The ICC densities for these animals were compared to that of the remaining animals in the colic group. This demonstrated no significant difference in ICC density in the myenteric region ($p=0.569$), the circular muscle layer ($p=0.851$) or of the overall ICC densities ($p=0.796$) between these two groups.

Inter-observer variability of grades given was also assessed in order to evaluate the reliability of semi-quantitative assessment. There was no significant difference between

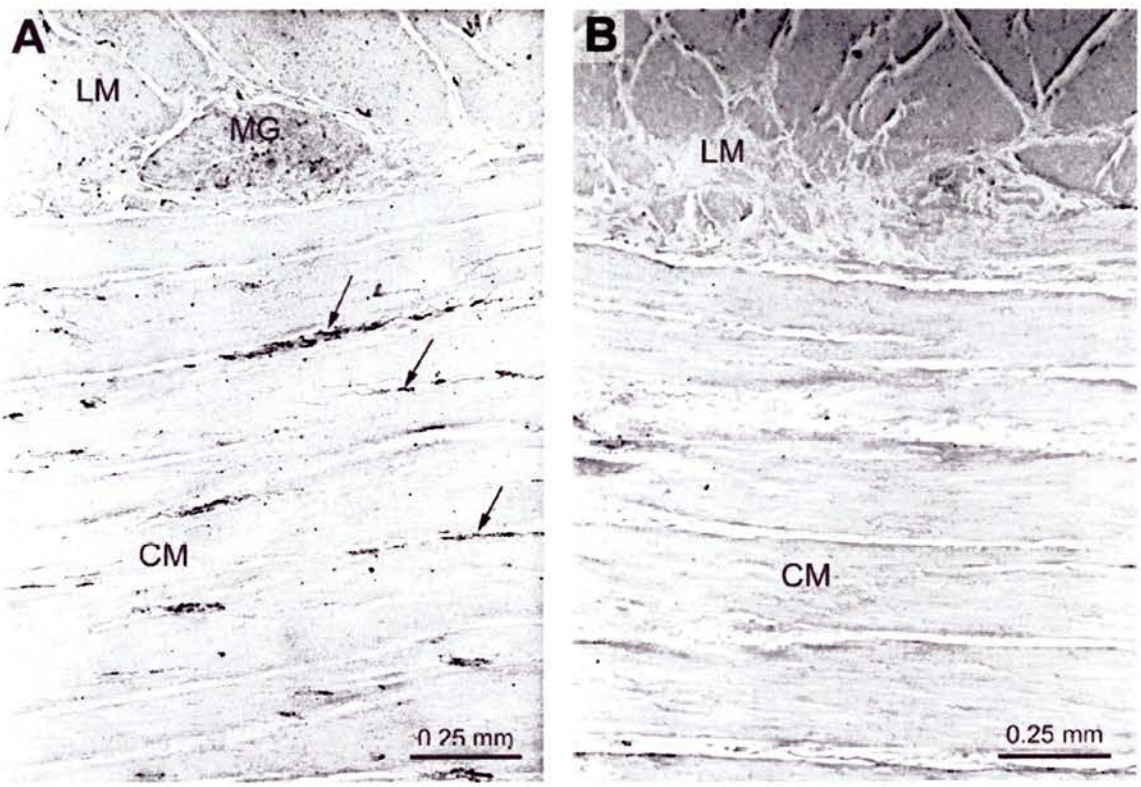


Figure 15: Section of a pelvic flexure from a normal horse (A) and one with a large colon torsion (B). c-Kit-immunoreactive ICC (arrows) can be observed throughout the circular muscle layer in A, but no detectable immunoreactivity is evident in B. LM: longitudinal muscle layer; CM: circular muscle layer; MG: myenteric ganglia.

the tissue grades allocated to the individual cases between the two independent observers ($p=0.609$) indicating good inter-observer agreement.

Evaluation of the H&E sections of the small intestine from affected horses revealed variable mild to moderate lymphocytic infiltration of the mucosa and submucosa which was not evident in the *muscularis externa.* Large colon samples from affected horses also revealed mild to moderate monocytic infiltration of the mucosa and submucosa. The submucosa was sometimes mildly to moderately oedematous. In some areas of the *muscularis externa,* a mild perivascular lymphocytic infiltrate was evident which did not extend beyond the perivascular connective tissue. Occasional migrating neutrophils were also observed in the *muscularis externa*. In addition, there was no obvious infiltrate in the region of the myenteric plexus area in either the small or large intestinal samples.

In five cases with a strangulating obstruction of the small intestine, c-Kit immunoreactivity was examined also in the grossly compromised segment of small intestine. Reduced ICC density was noted compared to the non-ischaemic tissue at the margin of resection from the same animal. However, based on the gross degree of tissue degradation assessed by H&E stain, these samples were not included in the statistical analysis.

2.3.1.3 ICC in recovered chronic EGS cases

As this study included only 2 animals, it was not possible to perform a meaningful statistical analysis. However, as a number of normal animals had been analysed in the previous study, it was considered appropriate to draw some general comparative conclusions from the current samples based on this. A continuous network of ICC was observed in the myenteric plexus region (Figure 16) consistent with a moderate density of c-Kit immunoreactivity as described above. A mixture of bipolar and stellate-shaped ICC was observed throughout the circular muscle layer of the *muscularis externa* again consistent with a moderate density grading. The orientation of the ICC processes was parallel to that of the smooth muscle fibres (Figure 17). The distribution and density of c-Kit-immunoreactive ICC were similar for both cases included in the study.

H&E evaluation of tissue sections (jejunum and ileum) as well as of cranial cervical and mesenteric ganglia in the both horses were performed. The number of neurons in the submucous and myenteric plexuses in a complete transverse section of the jejunum was evaluated in the first horse only and was found to be within normal limits as described by Doxey *et al.* (1995). However, the number in the submucous plexus of a complete transverse section of the ileum was reduced as were those in the ileal myenteric plexus. Neuronal morphology in the cranial cervical and cranial mesenteric ganglia in both horses appeared normal. However, subjective assessment suggested a moderate decrease in the number of neurons accompanied by an increase in cells resembling glial cells in these supporting the initial diagnosis of EGS.

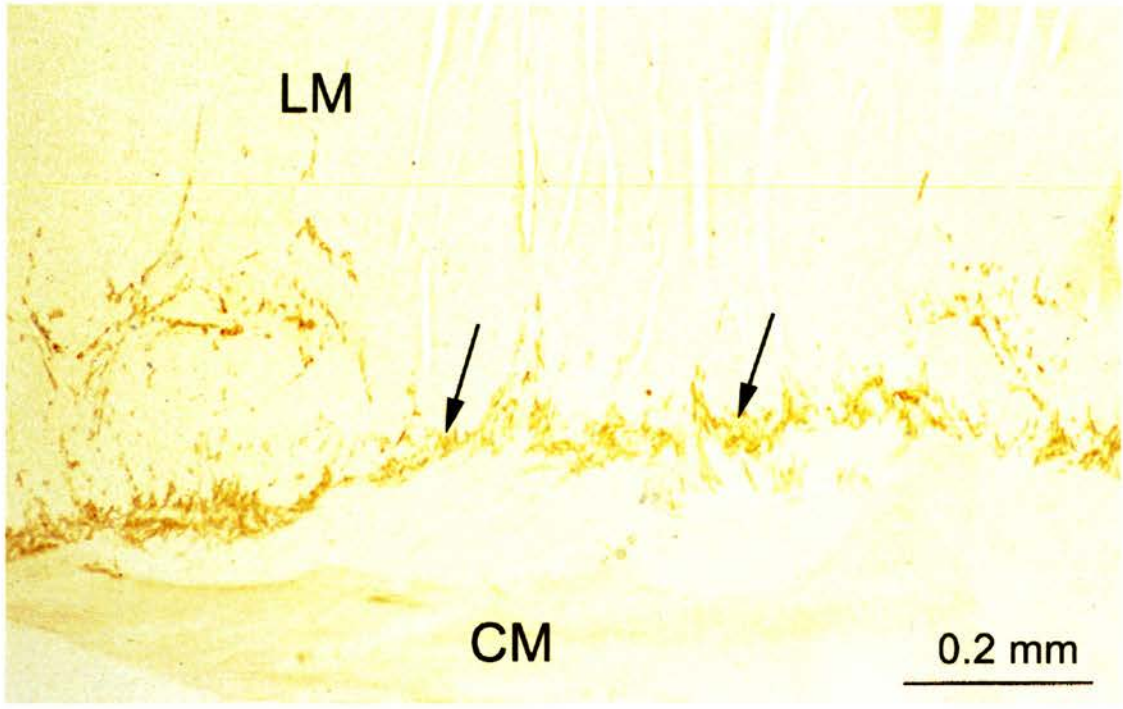


Figure 16: Myenteric plexus region of the ileum of one recovered EGS horse. A continuous band of c-Kit-immunoreactive ICC can be observed (arrows). LM: longitudinal muscle layer; CM: circular muscle layer.

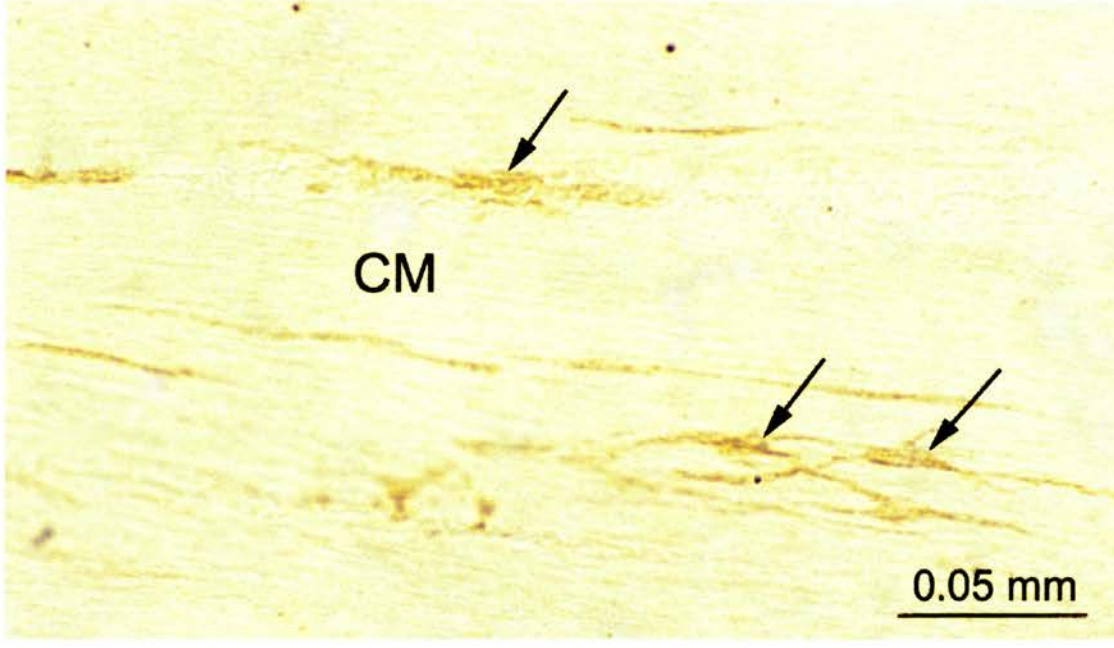


Figure 17: Circular muscle layer of the ileum of one recovered EGS horse. Individual c-Kit-immunoreactive ICC (arrows) could readily be observed throughout this muscle layer. CM: circular muscle layer.

2.3.1.4 ICC in the donkey

Immunohistochemical labelling of the tissue samples indicated that the density and pattern of c-Kit-immunoreactive ICC were very similar to that of the adult horse. As only a relatively small sample group was analysed, some caution in interpretation of the data is warranted. Although all diseased animals were diagnosed with a pelvic flexure impaction a small intestinal sample (ileum) was also collected and analysed in all animals apart from Donkey 12. The pattern of distribution in the small intestine of all donkeys was similar to that of the adult horses with the presence of a dense band of c-Kit-immunoreactive ICC along the myenteric plexus region (Figure 18). As in the horse, it was difficult to identify the shape of individual ICC present in this region. No significant difference in the density of ICC in the myenteric plexus region between the small intestinal samples collected from diseased animals and that of the controls ($p=0.4113$) was observed.

As for the myenteric plexus region, there was no significant difference in the ICC density between these two groups in the circular muscle layer ($p=0.7150$) of the small intestine. As observed in the adult horse, there was a mixture of both bipolar and stellate-shaped ICC with the cellular processes orientated parallel to that of the muscle fibres. In some animals, there appeared to be a band of stellate or bipolar-shaped cells located at the submucosal border of this muscle layer. This appeared to be located more closely to this inner border than that observed in the horse where the inner third of the muscle layer was involved.

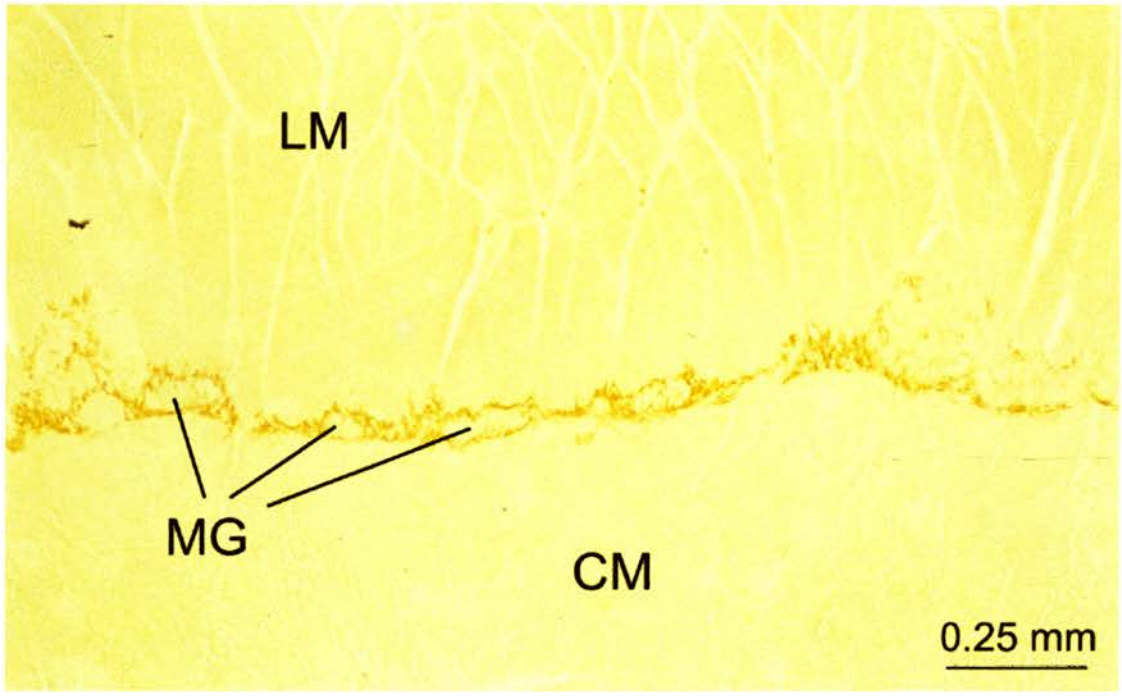


Figure 18: Myenteric plexus region of the ileum of a normal donkey. A continuous band of c-Kit-immunoreactive ICC can be observed. LM: longitudinal muscle; CM: circular muscle layer; MG: myenteric ganglia.

When the grades of the myenteric plexus region and circular muscle layer were analysed together, again there appeared to be no significant difference in the degree of immunoreactivity between the two groups of animals ($p=0.8551$).

In the samples collected from the large intestine, the pattern of immunoreactivity was similar to that of the adult horse, with a rather delicate lace-like pattern of ICC surrounding the myenteric ganglia and myenteric plexus region (Figure 19). Despite this delicate pattern, again it was difficult to identify individual ICC. There was no significant difference in the ICC density in the myenteric plexus region between normal and diseased animals ($p=0.4712$). The grades allocated to the two sample groups are illustrated in Figure 20.

Similarly, there was no significant difference in the degree of immunoreactivity in the circular muscle layer between normal and diseased animals ($p=0.9362$) (Figure 21). As for the adult horse, a mixture of bipolar and stellate-shaped ICC was observed in this region with their cellular processes parallel to that of the circular muscle fibres. When assessing the combined grades given of the myenteric plexus region and circular muscle in part of the intestinal tract, no significant difference was noted ($p=0.8102$).

H&E examination of all tissue samples was performed. This revealed all samples to be of sufficient quality to be included in the study. Mild to moderated degree of mucosal and submucosal lymphocytic infiltration was evident in a number of samples. A number of eosinophils were also observed which did not appear to extend into the *muscularis*

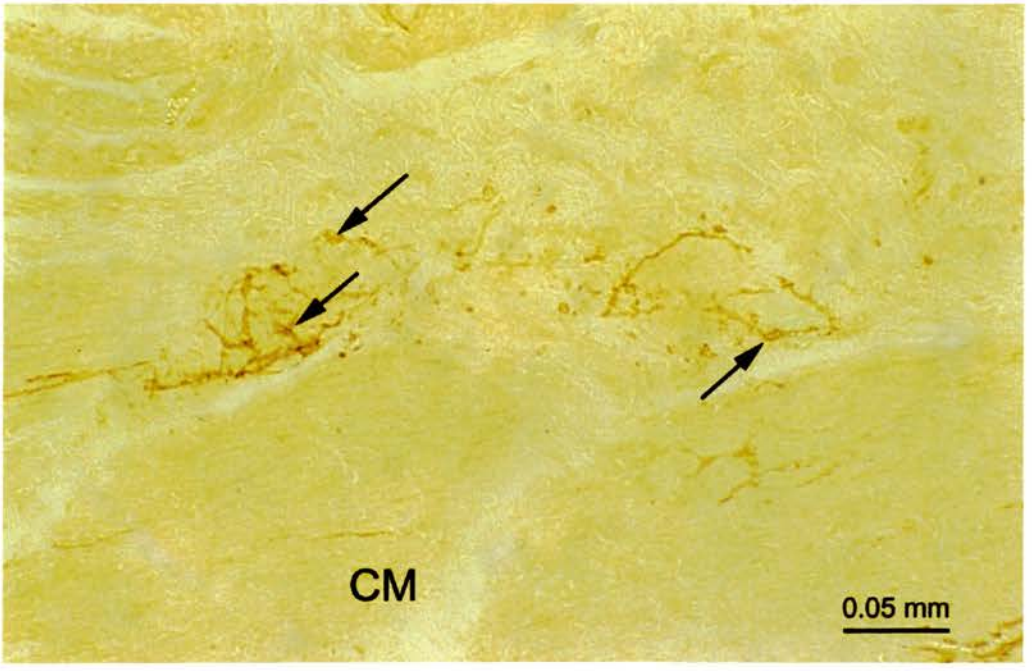


Figure 19: Myenteric ganglia of the pelvic flexure in an aged normal donkey. c-Kit-immunoreactive ICC form a lace-like pattern surrounding these (arrows). CM: circular muscle

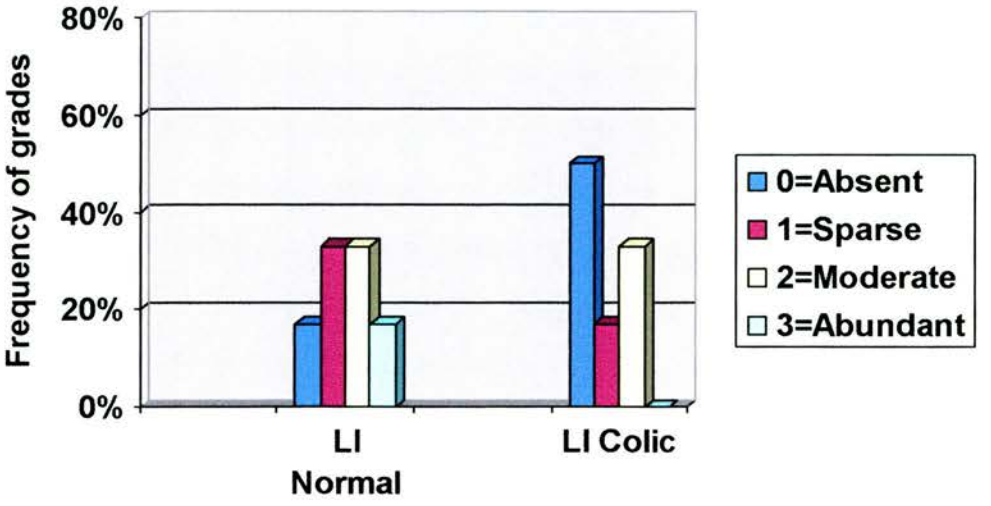


Figure 20: ICC density in the myenteric plexus region in the large intestine of normal donkeys (n=6) and donkeys with a large intestinal obstructive lesion (n=6). Frequency grade (%) represents the proportion of specimens that were assigned to a particular density grade: absent (0), sparse (1), moderate (2) and abundant (3).

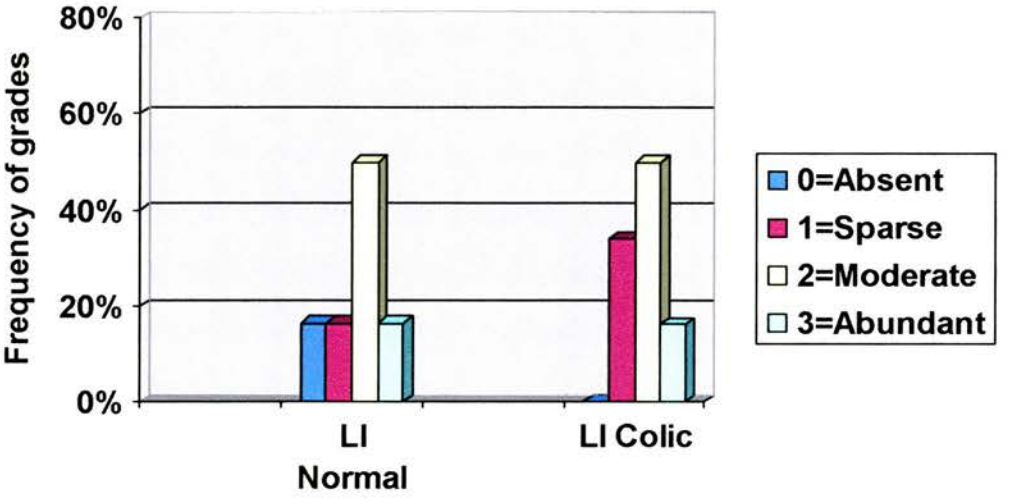


Figure 21: ICC density in the circular muscular region in the large intestine of normal donkeys (n=6) and donkeys with a large intestinal obstructive lesion (n=6). Frequency grade (%) represents the proportion of specimens that were assigned to a particular density grade: absent (0), sparse (1), moderate (2) and abundant (3).

externa. This infiltrate was observed in samples collected from both the small and large intestine in healthy and diseased animals.

Analysis of the condition of the teeth with respect to colic incidence failed to demonstrate a correlation between the two factors (p=0.5454, odds ratio=5). However, as for the c-Kit immunohistochemical data, it should again be emphasised that this study involved a relatively small sample size and so some caution in interpretation of the results is warranted.

2.3.1.5 ICC labelling: Vimentin

As this was a descriptive study, no statistical analysis was performed. The small intestinal samples displayed a similar pattern of immunoreactivity to that of c-Kit in the myenteric plexus region (Figure 22). However, as seen in this figure, the myenteric ganglia were also labelled. It was not possible to confidently identify individual ICC in the circular muscle layer using vimentin to target these cells. These findings meant that comparisons between normal and diseased animals were restricted to the myenteric plexus region. There did not appear to be any difference in the pattern or degree of vimentin immunoreactivity in this part of the small intestine.

In the large intestine, the myenteric ganglia appeared uniformly labelled as observed in the small intestine. However, it was not possible to observe labelled ICC in the myenteric plexus region between or surrounding the ganglia.

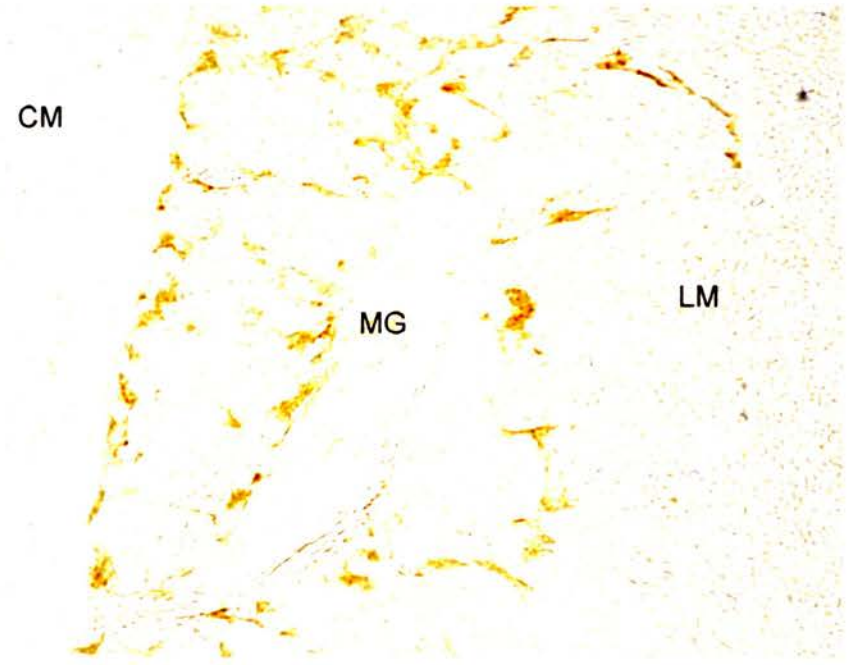


Figure 22A: c-Kit immunoreactivity in the myenteric plexus region of the ileum in a normal horse displaying typical ICC distribution. LM: longitudinal muscle layer; CM: circular muscle layer; MG: myenteric ganglia.

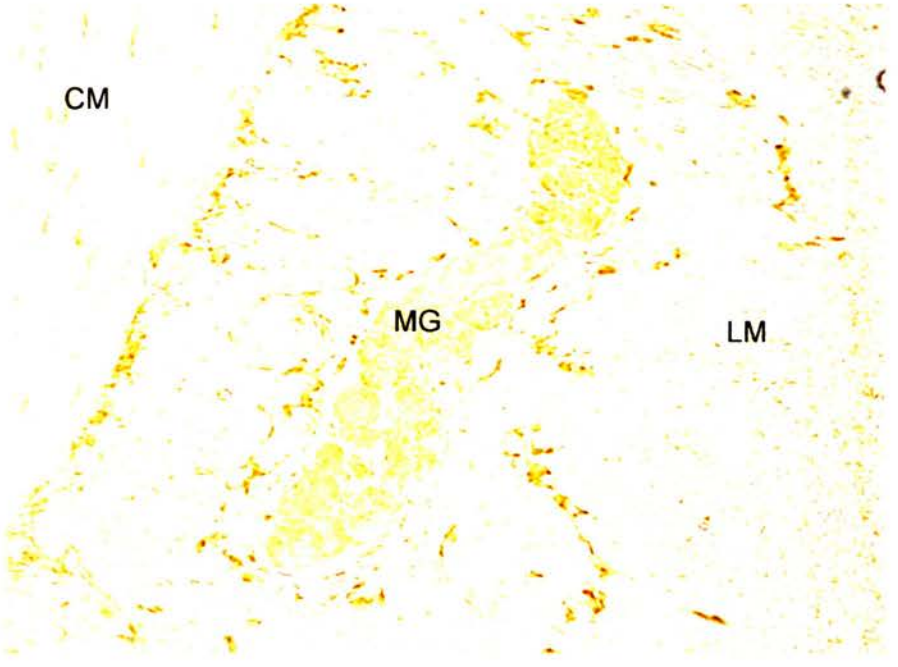


Figure 22B: Vimentin immunoreactivity in a subsequent section from the same animal (Figure 22A) demonstrating a distribution mirroring that of ICC. In addition, the myenteric ganglion has been labelled by vimentin. LM: longitudinal muscle layer; CM: circular muscle layer; MG: myenteric ganglia.

As noted in the small intestinal samples, it was difficult to make out individual ICC in the circular muscle layer using vimentin even in samples that had demonstrated an abundance of c-Kit-immunoreactive ICC.

2.3.1.6 Neuronal immunohistochemical labelling–PGP 9.5

This study compared the density of PGP 9.5 immunoreactivity in samples from normal and diseased horses, including the 2 recovered chronic EGS cases, as well as the donkey group. As a general comment that included both studies, the PGP 9.5 antibody displayed strong immunoreactivity and was easy to detect in the tissue sections. An example of this in the pelvic flexure of a control animal is illustrated in Figure 23. The orientation of the circular muscle layer was cut so that it was parallel to the muscle fibres. However, it was sometimes difficult to obtain true parallel sections, especially in the smaller pelvic flexure samples. Therefore, in a number of slides the nerve fibres would have been transected thereby showing punctuate positivity rather than a parallel linear positive reaction. In spite of this, it was still possible to estimate the degree of immunoreactivity due to the strong immunolabelling this antibody provides. Immunoreactive nerve fibres were detected in all layers of the intestinal tract. Ganglia were observed in the submucosa and myenteric plexuses. The size and number of ganglia varied of which the former was also clearly dependent on the angle of section. However, the ganglia observed in the myenteric plexus were larger and more numerous than those observed in the submucosa. Again because of the angle of sectioning, it was not possible to reliably count the individual cell bodies within the ganglia. Because only a limited amount of

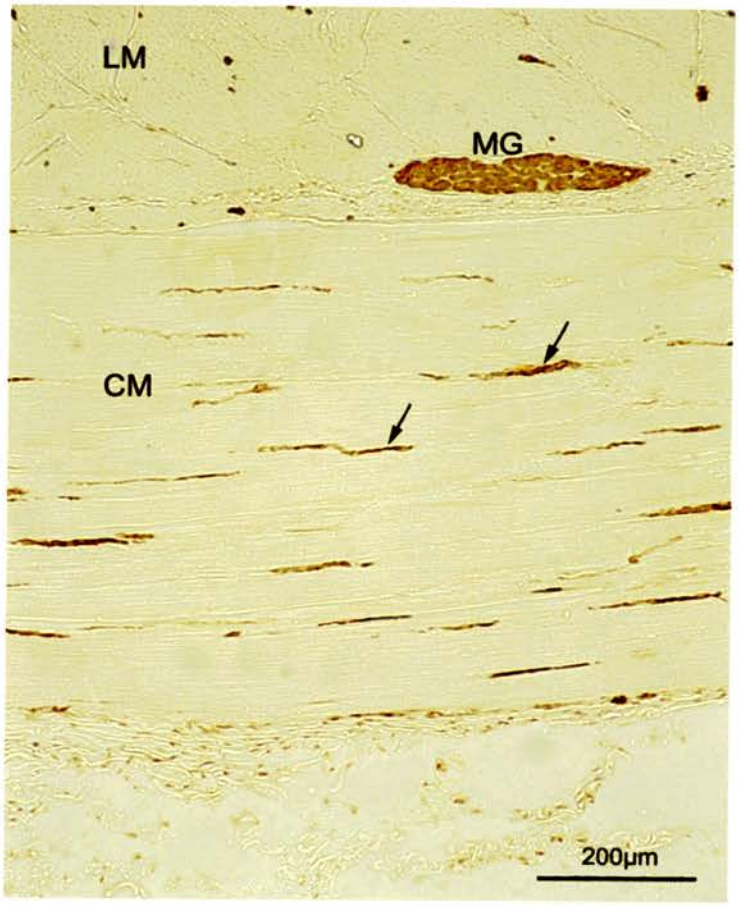


Figure 23: PGP 9.5 immunoreactivity in the pelvic flexure of a control horse. Arrows indicate labelled nerve fibres. Although only a limited area of the tissue section is seen in this picture, using the semi quantitative grading system described this horse was allocated a Grade 3 (abundant immunoreactivity) for both the myenteric plexus as well as the circular muscle layer. LM: longitudinal muscle layer; CM: circular muscle layer; MG: myenteric ganglia.

tissue was available and this did not always include mucosa, the PGP 9.5 density in this region could not be assessed in all cases.

In the equine study the PGP 9.5 density was assessed and compared in the mucosa, circular muscle layer and the myenteric plexus of both the ileum and pelvic flexure in normal and diseased animals. The p-values for these are described below (Table 6).

Table 6: p-values for PGP 9.5 density grades given in the horse groups.

	Normal vs. diseased Small intestine lesion	Normal vs. diseased Large intestine lesion
Mucosa	0.6278	0.1063
Circular muscle layer	0.3409	0.1880
Myenteric plexus	0.8375	0.9739

As seen from these data, there was no significant difference in the PGP 9.5 density in any of the anatomical areas in either of the groups. There were no animals with no detectable immunoreactivity although 2 animals with a large colon obstructive disorder had markedly reduced immunoreactivity throughout the pelvic flexure tissue sample (Figure 24) which was in stark contrast to that of the majority of animals, both normal and diseased horses.

There was no significant difference in the grades given by the observer on the two separate occasions ($p=0.7984$) indicating good grading consistency.

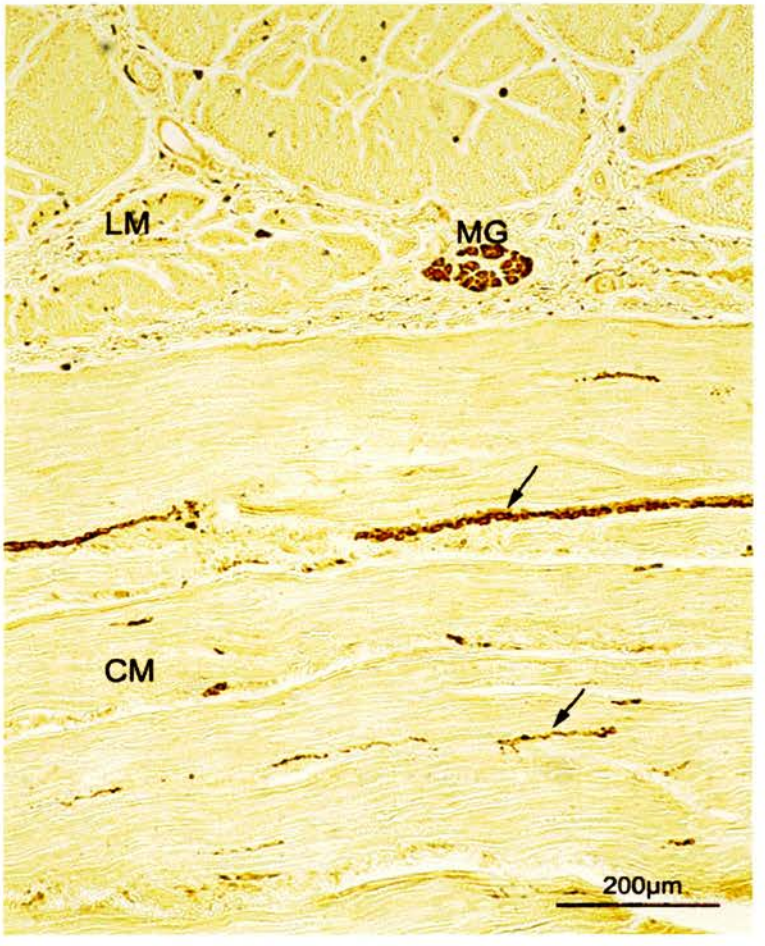


Figure 24: PGP 9.5-immunoreactivity in the pelvic flexure of a horse with an obstructive large colon disorder. Arrows indicate labelled nerve fibres. Although only a limited area of the tissue section is seen in this picture, using the semi quantitative grading system described this horse was allocated a Grade 1 (sparse immunoreactivity) for both the myenteric plexus as well as the circular muscle layer. LM: longitudinal muscle layer; CM: circular muscle layer; MG: myenteric ganglia.

Similar observations were made in the donkey groups as seen in Table 7. Both small and large intestinal samples from healthy and diseased animals were included in the statistical analysis. It is worth emphasising that although small intestinal samples from animals with a large colon impaction were evaluated, there was no evidence of systemic intestinal disease in these donkeys. This evaluation was merely performed for comparative purposes.

As for the horse study, there was good consistency (i.e. little variation) in the grades allocated to the tissue sections on the two separate occasions these were evaluated ($p=0.3410$).

Table 7: p-values for PGP 9.5 density grades given in the donkey groups.

	Normal vs. diseased Small intestine lesion	Normal vs. diseased Large intestine lesion
Mucosa	0.0578	0.6411
Circular muscle layer	0.3827	0.1706
Myenteric plexus	0.1904	0.3402

As for the ICC study in the 2 recovered EGS cases, descriptive observations only were made due to the small sample size. A limited number of PGP 9.5-immunoreactive ganglia were observed in the myenteric plexus. Occasional nerve fibres were observed in the myenteric plexus region as well as in both the longitudinal and circular muscle layers. No ganglia and few nerve fibres were observed in the submucosa as well as in the mucosa

(Figure 25) which differed markedly from that observed in the control animals from the colic study (Figure 26).

2.4 Discussion

This immunohistochemical study investigated various aspects of ICC development and distribution in health and disease. In addition, neuronal densities were evaluated and compared in normal and diseased horses as well as in donkeys.

2.4.1.1 Ontogeny of ICC

This study described the development and distribution of ICC in the equine foetus and neonate which has not been previously investigated. Developmental studies in other species have demonstrated that ICC start to colonise the intestinal tract about a third to half-way through gestation (Lecoin *et al.* 1996; Young *et al.* 1996; Torihashi *et al.* 1997; Kenny *et al.* 1999; Wester *et al.* 1999) and it seems reasonable to speculate that a similar time scale of development also occurs in the equine foetus. This would suggest that the initial colonisation of the equine intestinal tract would commence around the 4^{th} to 6^{th} month of gestation. However, considering the wide range of normal gestational lengths in this species (Rossdale *et al.* 1991; Rossdale 1993), there may also be some individual variation in the time of initial ICC colonisation. Unfortunately, equine foetuses under 6 months of gestation were not available for this study, principally because many early abortions are undetected.

Figure 25: PGP 9.5 immunoreactivity in the ileal mucosa of a horse recovered from chronic EGS. Grades were not allocated to the tissue samples in the two cases included in this study. However, very limited immunoreactivity is evident (arrows) and especially in comparison to the control horse in Figure 26.

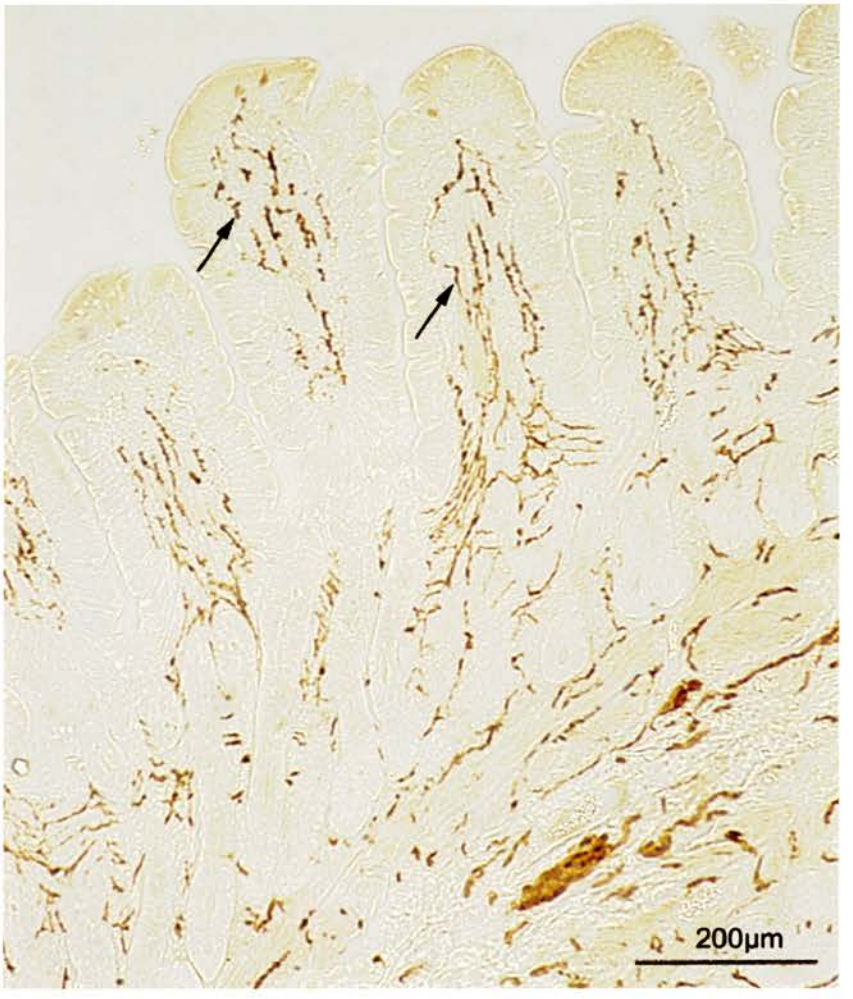


Figure 26: PGP 9.5 immunoreactivity in the ileal mucosa of a control horse. Labelled neurons are clearly visible in the lamina propria (arrows). Using the semi quantitative grading system described this horse was allocated a Grade 3 (abundant immunoreactivity) for this region.

At full term, ICC distribution and density in the small intestine appeared to be well developed. The cells were detectable not only in dense networks in the myenteric plexus region but also in the circular muscle layer of the *muscularis externa.* This distribution differs somewhat from that of other species where ICC in the circular muscle layer were only sparsely, if at all detectable until the neonatal period (Faussone-Pellegrini 1984; Young *et al.* 1996; Torihashi *et al.* 1995; Torihashi *et al.* 1997; Ward *et al.* 1997; Kenny *et al.* 1999; Wester *et al.* 1999).

As described in the previous chapter, different groups of ICC located at different anatomical regions of the GI tract, are thought to perform different functions (Sanders 1996). It has been demonstrated that ICC located around the myenteric plexus in the small intestine are involved in generating pacemaker activity in this region, while those located in the circular muscle layer primarily mediate neurotransmission (Sanders 1996). Assuming this also applies to the horse, the findings in the current study suggest that, in the equine foetus, development of ICC classes of both the pacemaker-generating as well as neurotransmission-mediating functions may be relatively advanced at the time of birth compared to other mammalian species. This apparent maturity is further supported by the observation that there did not appear to be significant changes in ICC density and distribution in the small intestine in the equine neonate compared to that of the full-term foetus. However, it is recognised that classification of the different types of ICC in the horse needs further work. For example, to investigate possible differences in function of the two morphological categories of ICC, it may be necessary to conduct both

electrophysiological and electron microscopical studies (Rumessen *et al.* 1993; Liu *et al.* 1998).

The maturity of small intestinal ICC networks at full term differed somewhat from that of the large intestine. In the latter, a proximal to distal gradient of ICC distribution was evident with the most distal part of the large intestine showing some evidence of ongoing development into the neonatal period. This type of developmental gradient has also been recognised in previous studies in other species (Faussone-Pellegrini *et al.* 1996; Ward *et al.* 1997; Kenny *et al.* 1998a). Ward *et al.* (1997) demonstrated that this developmental pattern also mirrored the onset of electrical rhythmicity in the murine gastrointestinal tract providing further evidence that some of these cells generate pacemaker activity.

At full term, in the most distal part of the large intestine, there was incomplete ICC colonisation of the innermost aspect of the circular muscle layer. Faussone-Pellegrini (1987) demonstrated in an ultrastructural study that the innermost aspect of the circular muscle layer in the mouse colon was the least differentiated layer at the time of birth when fibroblast-like cells were still differentiating into smooth muscle cells. Lecoin *et al.* (1996) subsequently demonstrated that ICC were mesenchymal in origin and hence share a common developmental origin with smooth muscle cells and fibroblasts. Indeed, this close relationship was further demonstrated when Torihashi *et al.* (1999) noted that blockade of c-Kit signalling induced transdifferentiation of ICC into a smooth muscle phenotype indicating that this signalling was important not only for differentiation but also for maintenance of the ICC phenotype. Considering this information, it is possible

that ongoing differentiation through c-Kit development and signalling is taking place in this last part of the large intestine and is not complete until some time after birth. In the 2 neonatal animals examined, there appeared to be a uniform distribution of ICC throughout the circular muscle layer in the most distal part of the large intestine indicating that this differentiation may be complete at this stage. The apparent immaturity at full term of the ICC in this region coupled with the proposed location of the main pacemaker-generating ICC at the submucosal border of the circular muscle layer in the large colon (Durdle *et al.* 1983; Ward and Sanders 1990) offers possible insight into the causes of equine neonatal intestinal obstructive disorders such as meconium impaction. Assuming this area is also the primary pacemaker-generating region in the horse, this immaturity of ICC at birth may contribute to retention of meconium in the distal colon and rectum by some neonatal foals. However, it is possible that even immature cells may have functional properties as demonstrated by Torihashi *et al.* (1997) and hence functional electrophysiological studies are needed in order to determine the onset of normal motility patterns, as well as confirming the location of primary pacemaker sites in the distal large intestine of the foal. Additionally, this highlights the need for an alternative immunohistochemical ICC marker as it is possible that ICC were present but that the c-Kit receptor was not fully developed and hence not recognised by these antibodies. As unsatisfactory results were obtained using the intermediate filament marker vimentin in the circular muscle of both the small and large intestine of adult horses, this labelling option was not further explored in the equine foetuses in the current study.

In the large intestine, there appeared to be an increased density of ICC in the circular muscle layer as well as in the myenteric plexus region in the areas near the taenial bands irrespective of gestational age or anatomical region. Increased ICC density in these areas has been described previously in adult horses (Hudson *et al.* 1999). The taenial bands are important as they are likely to be actively involved in peristalsis (Burns 1992). A second pacemaker-generating area in the myenteric plexus region of the colon has been demonstrated in the dog and rat initiating MPOs that spread into both the circular and longitudinal muscle layers (Smith *et al.* 1987b; Pluja *et al.* 2001). It is possible that ICC surrounding the myenteric plexus, especially in the region of the taenial bands, represent additional pacemaker-generating areas in the equine colon in addition to those proposed to be located in the circular muscle layer. This may account for the increased ICC density in these areas. A more likely explanation is that these ICC are involved in mediating neurotransmission (Thuneberg 1982; Daniel and Posey-Daniel 1984). The high ICC density in the region of the taenial bands may therefore be related to the dense innervation that has been noted in this area (Burns 1992).

In summary, this study has demonstrated the presence and distribution of ICC in the equine foetus during the latter half of gestation and into the neonatal period. It has shown that ICC distribution and density in the small intestine in the full-term foetus appear similar to that of the neonatal animal. However, it appears that ICC development in the distal large colon continues after birth with further colonisation of the inner aspect of the circular muscle region. This study has provided information on ICC development and

distribution in the equine foetus and neonate that will provide a foundation for future studies in diseased animals.

2.4.1.2 ICC colic study

Interesting observations were also made in the second immunohistochemical study comparing ICC density in tissue samples collected from normal and diseased adult horses. A significant reduction in ICC density was evident in pelvic flexure samples from animals with a large colon obstructive disorder compared to the control horses. This reduction included both the myenteric plexus region as well as the circular muscle layer. Little is known about the processes that lead to the loss of ICC in clinical disorders of GI motility (Sanders *et al.* 1999). Both hypoxia and inflammation have been implicated as possible causes of damage to these cells. Christensen and Rick (1994) suggested that ICC were susceptible to hypoxia by demonstrating an increase in intracellular vacuolation in response to hypoxia in the rat colon. However, they also noted that although changes to these cells occurred, the major ultrastructural features were preserved even in the tissues samples subjected to the most severe hypoxic environment (Christensen and Rick 1994). A parallel immunohistochemical investigation targeting the c-Kit receptor was not performed in their study. Although it is possible that a degree of hypoxia occurred to a proportion of horses with a large colon obstructive disorder in the current study, it seems unlikely that these were severe enough to account for the reduction in c-Kit-immunoreactive ICC observed. This is supported by the relatively normal gross

appearance of the intestine at surgery, and the fact that H&E assessment failed to demonstrate significant degenerative changes associated with hypoxia.

However, a mild to moderate inflammatory cell infiltrate of the mucosa and submucosa was observed in the majority of samples. Lu *et al.* (1997) demonstrated that an experimentally induced colonic inflammation resulted in reduced ICC densities in the dog. This noticeable disruption of ICC networks was most evident at the submucosal border as well as the most luminal part of the circular muscle region. This study demonstrated a range of ultrastructural changes to ICC including vacuolisation of the cytoplasm and blebbings of the plasma membrane, as well as signs of irreversible damage characterised by changes such as a large number of ruptured mitochondria and the depletion of many thin and intermediate filaments (Lu *et al.* 1997). The structural changes to ICC were accompanied by alteration of slow wave characteristics, including reduction in amplitude and duration of these, although the frequency appeared to be unaffected (Lu *et al.* 1997). It was proposed that these changes contributed to the suppression of spike-independent phasic contractions observed in this study (Lu *et al.* 1997). However, this assumes that ICC are indeed involved in these muscle activities. Spencer *et al.* (2003) demonstrated that electrical slow waves were not required for the generation of migrating motor complexes (MMC) in the mouse small intestine. These complexes were still present in W/W^V mutant mice, which lack pacemaker ICC and slow waves, and it was proposed that the generation and propagation of MMC were the result of an intrinsic capability of the ENS and not related to slow waves (Spencer *et al.* 2003). Nevertheless, it seems reasonable to speculate that changes in slow wave characteristics

may be an important factor in altered rhythmical contractile activity of the intestine. This is supported by the findings of another study where damage to ICC was experimentally induced in the mouse small intestine (Der *et al.* 2000). Changes to slow wave amplitude were again demonstrated, but in this study the frequency was also altered in some regions of the small intestine (Der *et al.* 2000). This alteration, where in some regions the frequency was reduced whereas in others it was doubled thereby abolishing synchronisation, was thought to result in the overall reduced transit of fluid in the proximal small intestine (Der *et al.* 2000). It is proposed that similar effects on equine slow wave patterns may have occurred in the current study, resulting in abnormal motility patterns. The majority of the inflammatory infiltrate was located in the mucosa and submucosa with only the occasional cell observed in the *muscularis externa*. The cells observed in the latter region were usually located around vessels in the myenteric plexus region. Assuming that the primary pacemaker generating area in the colon of the horse is also located at the submucosal border of the circular muscle layer, it is possible that these cells were affected by the nearby inflammatory process. However, there is a question of time. Der *et al.* (2000) demonstrated in their study that it took at least 18 hours for inflammation to cause disruption of ICC. The majority of animals in the current study underwent surgery well within this time scale, although of course, there could have been an ongoing intestinal inflammatory process prior to developing signs of colic and subsequent corrective surgery. This issue of whether the reduction in c-Kit-immunoreactive ICC happened during the current colic episode or sometime prior to it is especially relevant in view of the high proportion of horses with a history of previous colic episodes (15/31). This of course raises the question whether the colic episode

necessitating surgical correction was a consequence of, rather than the cause of the reduced ICC density. Previous studies have demonstrated that some horses suffering from one bout of colic are more likely to suffer further bouts (Ducharme *et al.* 1983; Cohen *et al.* 1995; Reeves *et al.* 1996, Cohen *et al.* 1999, Tinker *et al.* 1997a, b; Hillyer *et al.* 2002). For the majority of these animals, it was not possible to determine the reason for the recurrence of colic (Ducharme *et al.* 1983; Cohen *et al.* 1995; Reeves *et al.* 1996, Cohen *et al.* 1999, Tinker *et al.* 1997a, b; Hillyer *et al.* 2002). Schusser *et al.* (2000) demonstrated a significant reduction in myenteric neuronal densities in horses with acute and chronic obstructive disorders of the caecum and large colon, and it was proposed that these changes may have been a reason for the increased risk of recurrent colic from colonic or caecal dysfunction in these horses. It is also possible that at least some of the horses in the current study already had changes or disruptions to ICC networks making them more prone to developing colic. The capacity for ICC to repair following damage has only been partly explored. It appears that ICC can repair after mild to moderate injury although it may take up to 60 days to do so (Der *et al.* 2000; Chang *et al.* 2001; Wang *et al.* 2002). However, Der *et al.* (2000) did demonstrate irreversible changes to some ICC. With this information in mind, it seems reasonable to hypothesise that a proportion of cells may have been irreversibly injured during a previous inflammatory episode resulting in abnormal motility patterns. Against this argument, there was no significant difference in ICC densities in horses with a history of recurrent colic episodes compared to those without a known history of previous colic. However, this may be for the simple reason that the reduction in ICC in the group of horses without a history of previous colic

episodes happened during this occasion and that a proportion of these with irreversibly damaged ICC may go on to develop further colic episodes.

It is of course possible that there are other reasons why reduced ICC densities and disrupted networks could be present. This could include genetic abnormalities involving c-Kit receptor (Ward *et al.* 1994; Huizinga *et al.* 1995) or indeed its natural ligand SCF (Ward *et al.* 1995; Mikkelsen *et al.* 1998) both of which will affect ICC development as well as function.

No difference in c-Kit immunoreactivity was detected in horses with a small intestinal obstructive disorder. This finding was perhaps not too surprising considering that the original cause of colic in these animals could be explained by an external physical obstruction rather than by an intrinsic motility disorder. A mild to moderate inflammatory infiltrate of the mucosa and submucosa was also observed in these samples. However, these were some distance away from the pacemaker-generating ICC located in the myenteric plexus region, hence it seems unlikely that this would have affected the cells in this region. Similarly, the small intestinal samples included in the study were at the non-ischaemic margin of tissue resection and thereby would not have been exposed to significant hypoxic changes.

It is recognised that in 5 of the 10 cases involving sections of small intestine, jejunum rather than ileum was evaluated due to the anatomical location of the strangulating lesion. Although this differed from the location of the controls (ileum), an effort was always

made to evaluate the most distal segment of jejunum. Additionally, Hudson *et al.* (1999) described a similar pattern of ICC distribution in these two areas despite the fact that there appeared to be particularly strong immunoreactivity in the ileum. Considering the presence of strong immunoreactivity and a similar distribution of ICC in the jejunum of affected animals compared to control animals, it was considered a valid comparison despite the anatomical variation.

This immunohistochemical study has demonstrated a reduction in ICC density in horses with obstructive disorders of the large colon compared to control animals. Ultrastructural investigations of ICC under these conditions would offer further information on changes that happen under these conditions. Furthermore, functional electrophysiological and myoelectrical studies are needed in order to determine the precise effect reduced ICC density has on equine intestinal motility patterns.

2.4.1.3 ICC in recovered EGS cases

Equine grass sickness (EGS) is a frequently fatal disease of uncertain aetiology (Milne 1997; Cottrell *et al.* 1999). Recent evidence suggests that *Clostridium botulinum* type C toxin, acquired from soil while grazing, may be directly involved (Hunter *et al.* 1999; McCarthy *et al.* 2004). The main clinical signs described in these two chronic cases were related to dysfunction of the alimentary tract including pharyngeal and oesophageal dysphagia, colic and reduced GI motility. EGS is characterised by neuronal degeneration in the prevertebral and paravertebral autonomic ganglia and the enteric plexuses. The

degenerative changes in autonomic and enteric neurons comprise loss of Nissl substance resulting in cytoplasmic eosinophilia resembling chromatolysis, cytoplasmic vacuolation, eosinophilic spheroids within or adjacent to perikarya, nuclear pyknosis, margination or loss (Whitwell 1997) and reduction in the number of neurons (Doxey *et al.* 1995). In addition, neuronal chromatolysis occurs in autonomic and somatic lower motor neurons in specific brainstem nuclei, intermediolateral and ventral horns of the spinal cord and dorsal root ganglia (Hahn *et al.* 2001).

With experience, a clinical diagnosis can be very reliable (Milne *et al.* 1994) and the reversal of ptosis by the administration of phenylephrine eye drops provides useful supporting evidence (Hahn and Mayhew 2000). Examination of ileal biopsies obtained at laparotomy (Scholes *et al.* 1993) has proved a valuable technique and is currently the only confirmatory *ante mortem* diagnostic test, but is contraindicated in chronic cases where nursing is contemplated (Milne *et al.* 1994) and was therefore not undertaken in the present cases. Examination of the cranial cervical and cranial mesenteric ganglia gave a clear impression of reduced neuronal numbers, lending weight to the original clinical diagnoses.

Doxey *et al.* (2000) described the histopathological findings in the enteric plexuses, 11 months to 13 years after their initial illness, in four cases of EGS diagnosed on clinical grounds. In all four horses, there was a marked reduction in neurons in the jejunum and ileum, particularly the latter. However, three horses had been euthanased after an episode of acute colic which might have been attributed to a relapse. Recently, Owen and Kelly

(2003) described a complete clinical recovery in a case of EGS in which the disease was confirmed at the onset by jejunal and ileal biopsy. Despite the extensive neuronal depletion in the ileum seen in the current study, a continuous network of ICC was present in the myenteric plexus region in both animals. Numerous ICC were also observed within the circular muscle layer of the ileum. In a study by Hudson *et al.* (2001c), ICC were found to be significantly reduced in both the myenteric plexus and circular muscle regions of the ileum of horses with acute, subacute and chronic EGS compared to normal animals. The reduction of ICC in the circular muscle was greater in acute than chronic disease (Hudson *et al.* 2001c). The greater ICC density levels in chronic EGS cases and moderate levels in these recovered cases might be because the cells are not damaged to the same degree as in the more clinically-severe acute cases. Alternatively, the ICC may have been damaged but have started to recover. As described earlier in this chapter, a few studies have demonstrated that damaged ICC can recover or regain a functional phenotype, although it can take up to 60 days to do so (Der *et al.* 2000; Chang *et al.* 2001; Wang *et al.* 2002).

After recovering from the disease, one of the horses in the current study lived for 11 years without suffering a further episode of EGS or signs of impaired GI motility. The other animal remained clinically normal for 8 months before the oesophageal pathology necessitated euthanasia. One sample from a recovered chronic EGS case was also examined in the study by Hudson *et al.* (2001c) and was found to have no detectable c-Kit immunoreactivity. However, that animal, unlike the ones in current investigation, suffered a suspected "second episode" of EGS necessitating euthanasia, making it

difficult to determine if this reduction was a result of the subsequent disease or if it had reduced ICC prior to this episode.

In an *in vitro* electrophysiological study, it has been shown that in EGS, although the enteric neurons are severely damaged or depleted, the ICC-mediated control of electrical activity remains intact (Hudson *et al.* 2002). Although a contribution from the remaining neural components cannot be discounted, it seems most probable that the horses in the current study managed to maintain normal intestinal motility mainly by virtue of a functionally-intact network of ICC. However, examination of further recovered cases is warranted.

2.4.1.4 ICC in the donkey

This investigation set out to evaluate the distribution and density of ICC in the older donkey in the ileum and pelvic flexure as well as investigating if changes to these were present in animals with intestinal disease. Currently there is no information available on ICC in members of the Equidae family other than the horse. This pilot study demonstrated that the distribution and density of ICC in the donkey were similar to that of the adult horse both in the ileum and pelvic flexure. A dense, continuous band of ICC was observed along the myenteric plexus region of the small intestine making it difficult to identify individual cells. However, the ICC in this region appeared not to branch extensively into the longitudinal muscle layer as often observed in the horse. It is possible that this is an age change and younger donkeys should be examined in order to

clarify this point although of course, this may be a genuine difference between the horse and the donkey. Assuming the myenteric plexus region is also the pacemaker-generating area of the small intestine in the donkey, morphologically there appears to be no reason why this should not be functional in these aged animals. Similar observations were made in the circular muscle layer of the small intestine. Subjective assessment of the cells suggested that these appeared slightly more slender than that of the adult horse although the basic shapes (stellate and bipolar) were identical. As observed in some horses, there also appeared to be a band of ICC at the submucosal border of the circular muscle layer in the small intestine. This has not been reported in other species, although other species such as mice and humans appear to have an increased density of ICC at the deep muscular plexus region (Sanders 1996). It has been proposed that cells in this region may be able to generate slow waves (Hara *et al.* 1986). One way of determining this in the donkey (and horse) would be to carry out ablation experiments where the myenteric plexus region is separated from the remainder of the circular muscle and electrophysiological events are recorded.

The distribution and density of cells in the pelvic flexure region were very similar to that of the adult horse. This involved typical bipolar and stellate-shaped morphologies as well as the general distribution and density of ICC. Examination of samples collected from younger animals is needed to establish if ICC density or indeed distribution changes with increasing age. However, in view of the findings in this study it seems unlikely that this will be markedly different.

The second reason for carrying out this study was to perform a preliminary investigation into the possible involvement of ICC in the donkeys with intestinal disease. This was carried out because this particular herd of donkeys has a high incidence of impaction colic that coincides with winter housing (Duffield *et al.* 2002). However, the current study failed to demonstrate any significant differences in ICC density between the two groups although as a relatively small sample number was included some caution in interpreting these results is warranted. A possible correlation between colic incidence and the condition of the donkeys' teeth were also assessed. This was evaluated as it was proposed that the impaction colic diagnosed in 6 animals could be a result of other factors such as poor teeth rather than being caused by an intrinsic motility disorder. Again, no significant correlation between these two factors was determined. Although caution regarding the data analysis is warranted due to the small sample size, it seems reasonable to speculate that the problems experienced in this herd are not related to changes in ICC networks and densities. Other factors such as feeding and other husbandry practices should be further explored, emphasising the multifactorial causes of colic.

2.4.1.5 ICC labelling: Vimentin

This study demonstrated that the intermediate filament marker vimentin may provide an additional marker to c-Kit in the myenteric plexus region of the small intestine of the adult horse. The cytoplasm of animal cells is structured by scaffolding composed of actin microfilaments, microtubules, and intermediate filaments (Steinert and Roop 1988;

Fuchs and Cleveland 1998). Intermediate filaments are a diverse group of fibrous proteins expressed in the cytoplasm of higher eukaryotic cells (Fuchs and Cleveland 1998). This particular intermediate filament was chosen as developmental studies in the mouse intestine have demonstrated that ICC retains the expression of vimentin, unlike another intermediate filament desmin, into the neonatal period (Torihashi *et al.* 1995, 1997). The labelling of ICC was most easily observed in the myenteric plexus region of the small intestine. This was likely because the density of ICC in this region was the highest. In contrast, it was difficult to observe individual ICC in the circular muscle of both the small and large intestinal samples, although a shadow-like appearance was evident in some cases. Unfortunately, vimentin is also part of the structural scaffolding of mast cells and so offers no advantage over c-Kit in this respect which also labels these cells (Izushi *et al.* 1992; Galli *et al.* 1993). In spite of these limitations, vimentin could represent a useful additional marker of ICC in the myenteric plexus region of the small intestine. Both clinical and normal samples were assessed, but as the clinical samples also had normal c-Kit immunoreactivity, further studies are needed to investigate the use of vimentin as an alternative ICC marker. This is particularly true where there is reduced c-Kit immunoreactivity where an alternative marker could offer further information on the presence or absence of ICC in these cases.

2.4.1.6 Neuronal immunohistochemical labelling–PGP 9.5

These studies were carried out as part of the further investigation into the possible causes of equine intestinal motility disorders. EGS is perhaps one of the more

recognised conditions in the horse where intestinal dysfunction is linked to neuronal pathology (Scholes *et al.* 1993; Doxey *et al.* 1995; Whitwell 1997). Two previous studies have also demonstrated a significant reduction in myenteric neuronal densities in horses with acute and chronic obstructive disorders of the caecum and large colon (Schusser and White 1997; Schusser *et al.* 2000). Similarly, Burns *et al.* (1989) demonstrated a reduction in the myenteric neurons of the small colon in a mare with chronic intestinal pseudo-obstruction. These investigators proposed that the changes observed were linked to the intestinal motility disorders of these animals (Burns *et al.* 1989; Schusser and White 1997; Schusser *et al.* 2000). All the above mentioned studies were carried out using H&E evaluation of the tissue samples. Investigators have also evaluated PGP 9.5 as a neuronal marker in human intestinal motility disorders. This protein is involved in the ubiquitin-proteasome system which is a major pathway for selective protein degradation (Hershko and Ciechanover 1992) thereby forming an essential part of the normal cellular processes. The human disorders where this protein has been used as part of the investigation include conditions such as Hirschprung's disease (Sams *et al.* 1992; Oh *et al.* 2002) and idiopathic megarectum and megacolon (Gattuso *et al.* 1996, 1997). These human clinical studies have demonstrated a significant reduction in labelled neurons in the affected areas of intestine as well as documenting that PGP 9.5 provides a reliable and sensitive way of identifying neurones in the intestinal tract (Sams *et al.* 1992; Gattuso *et al.* 1996, 1997; Oh *et al.* 2002). The results of these latter studies formed the basis for this particular investigation. Overall there was no significant reduction in the neuronal densities in normal versus diseased animals. However, there were two horses with a large colon obstructive disorder (left

and right large colon displacement respectively) that did have a marked reduction in PGP 9.5 immunoreactivity. Interestingly, one of these horses had a history of previous colic episodes including surgery and it seems reasonable to speculate that this could be part of the disease process as proposed by Schusser *et al.* (2000) in their earlier study. It is worth mentioning that these two horses also had a significant reduction in ICC density that could also have contributed to the motility problems.

In the donkey study there were no animals with a marked reduction in neuronal density in either of the two groups indicating that disruptions to the ENS were not involved in the disease process. Furthermore, it demonstrated that PGP 9.5 remains strongly immunoreactive also in the older animal.

Labelling with PGP 9.5 in the recovered EGS horses demonstrated reduced immunoreactivity in both animals which was most marked in the ileal submucosa and the mucosa, although also noticeable throughout the remainder of the intestinal wall. The observations correspond well with the findings of Kitamura *et al.* (1998) who described a significant reduction in the PGP 9.5 immunoreactivity of EGS cases compared to normal control animals. This reduction in neuronal elements appeared to be more marked in chronic compared to acute cases and was more apparent in the submucosa than the myenteric region (Kitamura *et al.* 1998). However, no recovered EGS cases were included in this study. As already concluded in the above section, it is likely that the horses in the current study managed to maintain normal intestinal motility patterns due to an intact ICC network.

In summary, the different studies in this chapter have described the distribution and densities of ICC as well as enteric neurons in the intestinal tract of normal and diseased horses and donkeys using immunohistochemical labelling techniques. Changes to ICC networks were observed in a number of animals with intestinal disease suggesting that further investigations are warranted. These include *in vitro* intracellular electrophysiological studies in order to obtain functional information on slow wave activity in the large colon in normal and diseased animals as well as further investigation of the c-Kit receptor protein in health and disease. The latter is addressed in the following chapter where the level of expression of the gene encoding this protein is explored in normal versus diseased animals.

3 MOLECULAR STUDIES

3.1 Aims

Many cellular decisions concerning survival, growth and differentiation are reflected in altered patterns of gene expression (Zamorano *et al.* 1996). As part of the current investigation into the involvement of ICC in equine intestinal motility disorders, c-*kit* gene transcription was targeted. This gene was chosen in view of the functional importance of the protein product, c-Kit, in development and maintenance of the ICC phenotype (Ward *et al.* 1994; Huizinga *et al.* 1995; Torihashi *et al.* 1995, 1999). It was proposed that the degree of c-Kit immunoreactivity reflects the level of c-*kit* gene expression.

In order to test this hypothesis, c-*kit* and *β-actin* were identified from intestinal tissue samples from normal horses using polymerase chain reaction (PCR) amplification as described by Saiki *et al.* (1985, 1988) and Hart *et al.* (1988). The latter of these two genes, *β-actin*, is constitutively expressed encoding cytoplasmic microfilaments in eukaryotic cells (Romans *et al.* 1995) and was chosen as the control gene. Once the two genes were identified, transcription levels of c-*kit* and *β-actin* were quantified and compared between healthy and diseased horses using real-time reverse-transcriptase polymerase chain reaction (RT-PCR) analysis. These findings were compared to the density of c-Kit-immunoreactive ICC in the same horses in order to determine the correlation between c-*kit* transcription levels and c-Kit immunolabelling.

3.2 Materials and methods

3.2.1 Animals and samples used in the study

Surplus intestinal tissue samples were collected during surgery from 18 horses (median age 11.5 years, mean 9.9 years, range 2-24 years) of mixed breeds undergoing exploratory laparotomy for colic. Intestinal tissue samples were also collected from 15 control horses (median age 12.5 years, mean 12.0 years, range 1-33 years) of mixed breeds immediately (within 1 hour) following euthanasia by intravenous administration of quinalbarbitone sodium BP (Arnolds).

A section of ileum (level with the midpoint of the ileocaecal fold) was collected immediately following euthanasia from 8 control horses (median age 11.5 years, mean 15.6 years, range 1-33 years) with no known previous history of colic. Surplus intestinal samples were also collected during surgery from 8 animals (median age 13.0 years, mean 14.0 years, range 8-24 years) with an obstructive lesion of the small intestine. Samples collected from this group included sections of jejunum or ileum from the non-ischaemic tissue at the margin of resection. Although an attempt was made to select tissue that was closest to or included ileum to match the control samples, this was sometimes not possible if the strangulating lesion involved jejunum only (n=5).

In the remaining animals, a section of pelvic flexure (located at the junction between the left ventral colon and left dorsal colon) was collected from 7 control horses (median age

11.0 years, mean age 11.0 years, range 3-19 years) and 10 horses (median age 6.0 years, mean age 6.7 years, range 2-14 years) with a large colon obstructive disorder. As for the small intestinal control samples, the pelvic flexure control samples were collected immediately following euthanasia from horses with no known history of previous colic episodes. Samples collected from the colic group were surplus tissue following a pelvic flexure biopsy submitted for histological analysis at surgery. All samples were collected with the owners' written consent.

Details on the different horses and diagnoses are described in Table 8.

From all tissue sections collected, a 0.5x0.5 cm full-thickness sample was cut and placed in an RNA-stabilising solution (RNA Later, Qiagen, Crawley, West Sussex, UK). Unless samples were immediately processed, they were subsequently frozen and stored at -70°C. The remaining intestinal tissues samples were placed in 10% buffered formalin for subsequent immunohistochemical analysis and fixed for at least 24 hours prior to processing.

Table 8: Details on horses included in the study.

Horse Group	Age (Yrs)	Sex	Sample	Reason for euthanasia/ diagnosis
Small intestine normal				
SSPCA	31	G	IL	Chronic orthopaedic disorder
03/488	11	G	IL	Chronic orthopaedic disorder
01/488	1	M	IL	Chronic orthopaedic disorder
02/252	33	G	IL	Chronic orthopaedic disorder
02/56	8	G	IL	Chronic orthopaedic disorder
Lamb	12	F	IL	Chronic orthopaedic disorder
01/462	11	G	IL	Chronic orthopaedic disorder
03/381	18	F	IL	Reproductive tract disorder
Small intestine colic				
00/656	8	F	IL	Mesenteric rent
02/90	12	G	JEJ	Epiploic foramen entrapment
09/06/03	19	G	JEJ	Strangulating lipoma
03/435	12	G	IL	Strangulating eosinophilic lesion
02/110	14	G	JEJ	Epiploic foramen entrapment
02/97	9	G	JEJ	Epiploic foramen entrapment
03/209	14	G	JEJ	Meckel's diverticulum
27870	24	F	IL	Strangulating lipoma
Large intestine normal				
Wispa	15	F	PF	Chronic respiratory disorder
99/788	19	G	PF	Chronic orthopaedic disorder
03/488	11	F	PF	Chronic orthopaedic disorder
03/303	3	G	PF	Chronic orthopaedic disorder
03/381	18	F	PF	Reproductive tract disorder
Wobbler	2	F	PF	Neurologic disorder
03/124	9	F	PF	Chronic orthopaedic disorder
Large intestine colic				
03/242	9	G	PF	Right large colon displacement
27644	5	G	PF	Left large colon displacement
03/340	8	G	PF	Right large colon displacement
18/05/03	6	G	PF	Right large colon displacement
Pedrita	2	F	PF	Large colon torsion
28570	3	F	PF	Left large colon displacement
28535	6	G	PF	Right large colon displacement
Annie B	9	F	PF	Left large colon displacement
28225	5	F	PF	Left large colon displacement
28287	14	F	PF	Large colon torsion

Key: G: gelding; M: male; F; female; Il: Ileum; Jej: jejunum; PF: pelvic flexure

3.2.2 Imunohistochemical labelling of tissue samples

Please refer to the previous chapter for details on processing of the samples for c-Kit immunohistochemistry.

3.2.3 Sample handling and processing prior to polymerase chain reaction (PCR)

The following protocol was used for sample preparation prior to PCR amplification:

3.2.3.1 RNA extraction

Safeguards are necessary during RNA extraction in order to reduce the risk of contamination with RNAse enzymes from the environment. RNAse-free microfuge tubes and pipette tips containing filters (Greiner Bio-One Ltd, Stone House, UK) were used for all procedures involving RNA and gloves were changed frequently. Additionally, all glassware was baked at 150°C for 2 hours and all bench surfaces, pulverisors, and scalpel blades were wiped with RNAzap (Qiagen) and rinsed in RNAse-free water prior to use in order to further minimise RNAse contamination.

From each intestinal sample collected, the mucosa was carefully dissected away and 30mg of remaining sample selected for further processing. The mucosa was removed as mast cells also express c-Kit (Galli *et al.* 1993) and the majority of these cells are located in this area. In this way, a possible overestimation of c-*kit* mRNA was avoided. Subsequent parallel H&E as well as toluidine blue-stained sections demonstrating mast

cell metachromasia confirmed that the vast majority of cells present were located outside (at the luminal side of) the *muscularis externa*.

RNA extraction was performed using the RNeasy Mini kit (Qiagen). The selected tissue sample was immediately placed in 600µl of a denaturing guanidine isothiocyanate (GITC)–containing buffer, which also inactivates RNAses and lyses the tissue to ensure isolation of intact RNA. Disruption of the tissue sample was carried out using RNAse-free plastic pulverisors. Once the sample was completely disrupted, it was homogenised by centrifugation at 12000 rounds per minute (rpm) for 3 minutes in a QIAshredder column (Qiagen). Following this step, 600µl of 70% ethanol was added to the lysate to provide appropriate conditions for binding to the column membrane. 700µl of this sample was subsequently centrifuged at 10000 rpm for 15 seconds in order to bind the RNA to the membrane. The flow-through was discarded and 700µl of a buffer RW1 containing a guanidine salt was added to the column before centrifuging this at 10000 rpm for 15 seconds. The column was transferred to a fresh collection tube before adding 500µl of a buffer RPE containing 80% ethanol. This was subsequently centrifuged at 10000 rpm for 15 seconds. Once the flow-through was discarded, a further 500µl of Buffer RPE was added and centrifuged at 10000 rpm for 2 minutes in order to dry the column membrane. The isolated RNA sample in the membrane was eluted in 30µl RNAse-free water (Sigma Aldrich) and, unless further processed, immediately stored at -70°C.

3.2.3.2 Confirmation of RNA quality

To verify the integrity of extracted RNA, a 3µl sample was mixed with 7µl of loading buffer (Appendix 2) and run on a 1% agarose gel at 70-80V for 30-45 minutes. All electrophoresis equipment (gel tanks, casting trays and combs) used for RNA analysis was washed with RNAzap (Qiagen) and rinsed with RNAse-free water before use. The gel was made by melting 1g of agarose (Roche Diagnostics, Lewes, UK) in 100ml of 1xTAE buffer (Appendix 3) in a microwave. Following cooling the dissolved agarose to 'hand-hot' temperature by holding the flask under running tap water, 1µg ml^{-1} ethidium bromide (EtBr) (Sigma Aldrich) was added. The agarose solution was poured into a gel casting tray with a comb inserted and allowed to set. The gel was placed in an electrophoresis tank, covered with 1xTAE buffer, and the combs carefully removed before pipetting the RNA product into the appropriate well. The integrity and size distribution of the total RNA extracted was evaluated and based on the appearance of the respective ribosomal bands. These should appear as sharp bands, the 28S ribosomal RNA bands present with an intensity approximately twice that of the 18S RNA band. Prominent smearing between these bands would have been indicative of significant degradation of the RNA sample during collection or preparation. However, all samples included in this study (n=33) fulfilled the criteria of good quality RNA.

3.2.3.3 cDNA synthesis

First-strand complementary DNA (cDNA) synthesis was carried out using the Qiagen Omniscript Reverse Transcriptase kit (Qiagen). An oligo-dT primer (Roche Diagnostics)

was used with a reverse transcriptase (RT) enzyme to initiate the cDNA synthesis from polyadenylated (polyA$^+$) RNA according to the manufacturer's instructions. Estimation of RNA concentration had been attempted by measuring the absorbance at 260 nm (A_{260}) in a spectrometer. However, following repeatedly unreliable estimation of RNA concentration using this method, it was decided to use a standard volume of 5µl for each reaction which was considered to be within the recommended RNA concentration for this kit (50ng-2µg RNA). This method was considered acceptable as quality control of extracted RNA for each animal had already been carried out as previously described. Furthermore, as described in section *3.2.5.1*, quantification using real-time PCR was based on a ratio between the target gene, c-*kit*, and the chosen housekeeping gene, *ß-actin*, for the individual animal so the actual amount of starting material was not critical in this case.

The following RT reaction was set up and incubated in a water bath at 37°C for one hour: 5µl total RNA, 2µl deoxynucleotide triphosphates (dNTPs, 5mM each dNTP) (Roche Diagnostics), 2µl oligo-$dT_{(25)}$ primer (10µM), 2µl first-strand buffer, 1µl reverse transcriptase enzyme and 8µl of RNAse-free water to give a total reaction volume of 20µl. The synthesised cDNA preparations were used immediately or stored at -20°C for subsequent use.

3.2.4 Polymerase chain reaction (PCR)

The methods used in this investigation derive from those described by Saiki *et al.* (1985, 1988) and Hart *et al.* (1988). They utilise the principle of using sequence-specific oligonucleotide primers to specifically amplify a cDNA sequence from genes of interest, in this case c-*kit* and *β-actin*. The first step in this process is the design of the appropriate oligonucleotide primers and is described below.

3.2.4.1 Primer design for c-kit and β-actin for first-round and nested PCR reactions

External and internal primers for both c-*kit* and *β-actin* (Table 9) were designed using the incomplete equine mRNA c-*kit* sequence and complete equine *β-actin* mRNA sequence (Accession number AF055037 and AF035774 respectively, available in NCBI Sequence Viewer, NCBI, GenBank database) using the Primer3 Output programme (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). All primers were designed to ensure there was no sequence complimentarity within or between primer pairs to avoid self-annealing. Oligonucleotides were synthesised by Qiagen and diluted to 100ng/µl with PCR-grade water for storage in aliquots at -20°C to avoid degradation or contamination.

Table 9: Oligonucleotides for amplifying segments of equine c-*kit* and *β-actin*.

	DNA sequence	Orientation	PCR Annealing temp (°C)	Function	Amplicon length (bp)
Equine c-*kit*	CCC ACC AAG ACA GAC AAG AG	Forward	55°C	Amplification internal	293
	CGA GCA TTT CCT TTG ACC AC	Reverse	55°C		
	ATC ACG CAG AAG CAG CAC	Forward	55°C	Amplification external	475
	TGC CAT CCA TTT CAC AGG TAG	Reverse	55°C		
Equine *β-actin*	TGG GCC AGA AGG ACT CAT AC	Forward	55°C	Amplification internal	500
	CTT GAT GTC ACG CAC GAT TT	Reverse	55°C		
	GTC TTC CCC TCC ATC GTG	Forward	55°C	Amplification external	640
	GCA GCT CGT AGC TCT TCT CC	Reverse	55°C		

[a] Oligonucleotide sequences are listed in the 5' to 3' direction.

3.2.4.2 Optimisation of PCR reaction conditions

Initial optimisation experiments were carried out to ensure that reaction conditions gave maximum efficiency and specificity to the amplification. The two most important factors tested were magnesium chloride ($MgCl_2$) concentration and annealing temperature of the PCR reaction. Initial PCR reactions were carried out on replicates of chosen control samples under standardised reaction conditions with titration of $MgCl_2$ concentrations. A standardised reaction concentration consisted of a PCR programme comprising a predenaturation step of 3 minutes at 94°C followed by 35 cycles, each consisting of 45 seconds at 94°C, 1 minute at 55°C, and 1 minute at 72°C, and a final extension step for 10 minutes at 72°C. The $MgCl_2$ concentrations tested were 1mM, 1.5mM, 2mM, 2.5mM and 3mM respectively. The optimal $MgCl_2$ concentration was determined subjectively

by viewing EtBr-stained gels of the final PCR product. The $MgCl_2$ concentration which gave the best combination of high sensitivity (bands visible at greater dilutions) and absence of non-specific bands was selected. Based on these criteria, a $MgCl_2$ concentration of 1.5mM was chosen for all subsequent PCR reactions.

The annealing temperature was evaluated by placing identical sets of reactions through identical PCR reactions, differing only in annealing temperatures (55°C, 57°C and 58°C respectively). The criteria for determining the optimal annealing temperature were a combination of reaction sensitivity and specificity as described. The annealing temperature finally chosen for both c-*kit* and *β-actin* was 55°C.

In spite of rigorous optimisation, a degree of primer-dimer formation occurred when amplifying the smaller c-*kit* product (293 base pairs). This was likely due to the small product size needed for subsequent real-time analysis, although mainly due to the actual mRNA c-*kit* sequence. This contains a high proportion of repetitive base sequences making optimal primer design difficult, especially for smaller products. Because of this, it was important to sequence the amplified product to ensure it was the targeted c-*kit* sequence. This is described further in the next section.

All PCR reactions were carried out in sterile 0.2ml PCR tubes (Greiner Bio-One Ltd) with a final reaction volume of 25µl. The basic components of the PCR reaction were as follows:

1x Taq PCR reaction buffer (Roche Diagnostics), 1.5mM $MgCl_2$, 200µmol dNTP (50µmol dATP, dCTP, dGTP and dTTP) (Roche Diagnostics), 1.5 Taq polymerase (Roche Diagnostics), 200pmol of c-*kit* and *β-actin* primers respectively and PCR-grade water (Sigma Aldrich). The PCR programme consisted of a predenaturation step of 3 minutes at 94°C followed by 35 cycles, each comprising a denaturation step of 30 seconds at 94°C, an annealing step of 30 seconds at 55°C, and an extension phase of 1 minute at 72°C, before a final extension for 10 minutes at 72°C.

Negative controls were included alongside each reaction to check for contamination by substituting cDNA for PCR-grade water.

3.2.4.3 Gel electrophoresis of PCR products

Once the PCR cycle programme was completed, 20µl of each sample was mixed with 4µl loading buffers/dyes (Appendix 2) in order to better visualise the migration pattern of the PCR products during the subsequent gel electrophoresis. A 100 base pair ladder marker (DNA molecular weight marker XIV, Roche Diagnostics) was run alongside the target products to size the PCR amplicons and hence identity of the amplified products. Inclusion of EtBR as described enabled visualisation and photo documentation of the products using ultraviolet light. The gel was electrophoresed at 75-80V for 45-50 minutes to resolve the amplified products. PCR reactions using both the external and internal primers were optimised separately prior to carrying out the nested reactions.

3.2.4.4 Sequencing of PCR products

In order to ensure that the correct products had been amplified, PCR products of correct size were excised from the agarose gel and purified using a gel extraction kit using a bind-wash elute method (MinElute Gel Extraction Kit, Qiagen). The gel slice containing the PCR product of interest was placed in a sterile 1.5ml eppendorf tube (Greiner) and buffer QG added to solubilise it (3 volumes buffer QG to 1 volume gel). The mixture was incubated at 50°C for 10 minutes in a water bath. An equal volume of 70% isopropanol (Sigma Aldrich) was added to the dissolved gel before loading the entire volume into a spin column and subsequently centrifuged at 12000 rpm for 1 minute. The nucleic acids were absorbed onto a silica-gel membrane in high-salt conditions provided by the buffer QG during this process. Once the flow-through was discarded, a further 500µl of buffer QG was added and spun at 12000 rpm for 1 minute. The flow-through was discarded and the sample spun again as described. Following this, 750µl of buffer PE was added to the column and centrifuged at 12000 rpm for 1 minute. Again, the spin was repeated as described. Finally, the now purified PCR product was eluted in 30µl PCR-grade water (Sigma Aldrich) for subsequent sequencing. Eluted PCR product was checked for product size and purity on an agaorose gel before proceeding to sequencing.

Sequencing reactions were carried out using 2µl PCR product as template together with 1.6pmol sequencing primer (c-*kit* and *β-actin* left primers for internal and external products as appropriate), 2µl BigDye v3.1 Sequencing Buffer and 4µl BigDye Terminator v3.1 Ready Reaction Premix (Applied Biosystems). The reaction volume was made up to a total 10µl with PCR-grade water (Sigma Aldrich). The sequencing

reaction consisted of a predenaturation step of 1 minute at 96°C followed by 25 cycles, each consisting of 10 seconds of denaturation at 96°C, 5 seconds of annealing at 50°C, and 4 minutes of extension at 60°C. The temperature was then held at 4°C until ready to send for sequencing at the ICAPB, Ashworth Laboratories (Kings Buildings, University of Edinburgh, Edinburgh, UK). Once the sequence identification was obtained, a database search was performed (http://www.ncbi.nlm.nih.gov/BLAST) which verified that the sequences obtained corresponded to c-*kit* and *β-actin* respectively for both the external and internal primers as well as for the nested product.

3.2.5 Quantitative real-time PCR

3.2.5.1 Principles

The real-time measurement of a gene product concentration during a PCR reaction is made possible through the incorporation of a fluorescent dye, SYBR Green, which binds specifically to double-stranded DNA. The fluorescence of this dye is greatly enhanced during binding and therefore during each annealing and extension phase of DNA synthesis. The fluorescent signal emitted during each cycle is detected by the optical system of the fluorimeter within the thermal cycler (Light Cycler™, Roche Diagnostics) and illustrated real-time. This is possible as all samples are loaded into glass capillaries (Light Cycler™ Capillaries, Roche Diagnostics) with a high surface-to-volume ratio that allows rapid detection of the fluorescent signal.

The quantification analysis is based on the principle that detectable fluorescence above a threshold value is directly proportional to the initial sample copy number. In other words, the higher the initial starting number of DNA copies, the fewer cycles it takes for the fluorescent signal to reach the detectable level. This principle is illustrated in Figure 27.

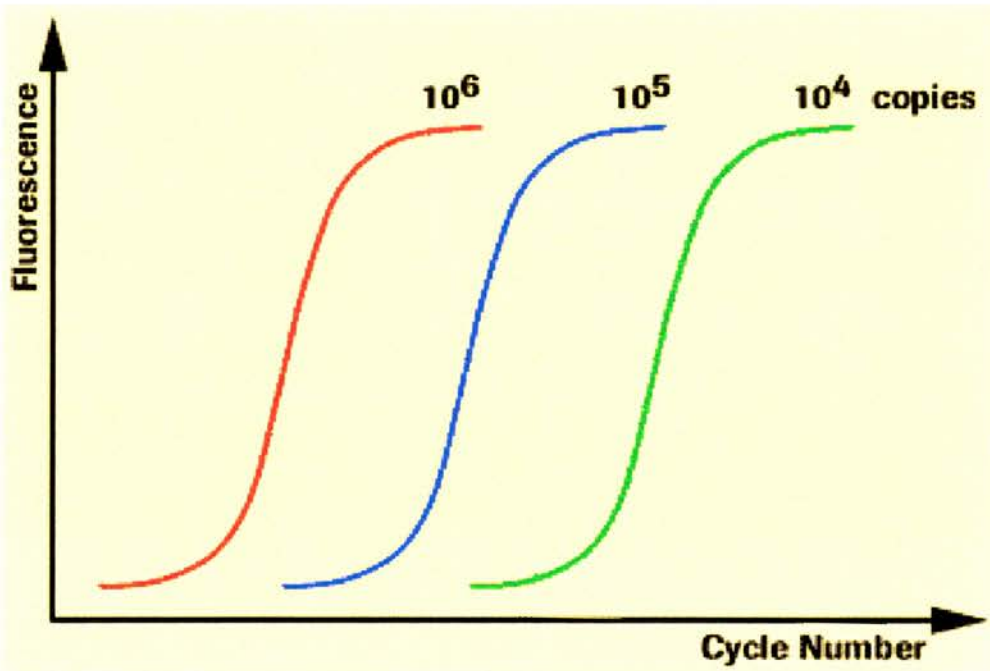


Figure 27: The principle of real-time quantitative RT-PCR: Detectable fluorescence above a set threshold value is directly proportional to the initial number of DNA copies, i.e. the higher the initial DNA copies, the fewer cycles are needed to reach the set threshold value (from Light Cycler™, Roche Diagnostics).

In order to determine the template concentration of unknown clinical samples, a standard curve had to be established. This was obtained through first amplifying the genes of interest, c-*kit* and *β-actin*, from a selected normal control animal through a nested PCR reaction as described. The purpose of doing a nested reaction was to amplify these genes to produce a range that would exceed any measured concentrations of the actual clinical samples. The undiluted PCR product was diluted in 10-fold serial dilutions to 10^{-12} with PCR-grade water to produce 13 samples that would serve as reference samples. These samples were amplified to produce a range of values based upon initial DNA concentration. The point where the threshold for fluorescent signal detection was obtained, the crossing line, was used to provide the relative value for each sample. These crossing point values for the set of standards, expressed as a cycle number, were plotted against a log concentration to give a standard curve. This principle is illustrated in Figure 28. Following amplification of clinical samples, relative values for starting template concentrations were obtained based on the standard curve values. The figures obtained are not absolute numbers of transcripts but relative amounts, since the number of mRNA copies in the standard dilution series is not known.

In order to standardise measurements between samples and to account for differences in RNA concentration and cDNA synthesis efficiency between samples, it was necessary to normalise the data. This was achieved through standardising values of the gene of interest (c-*kit*) to the expression of a continuously expressed housekeeping gene, in this case *β-actin*. The choice of this gene is addressed further in the discussion.

All analyses of transcript levels were made using figures normalised in this way.

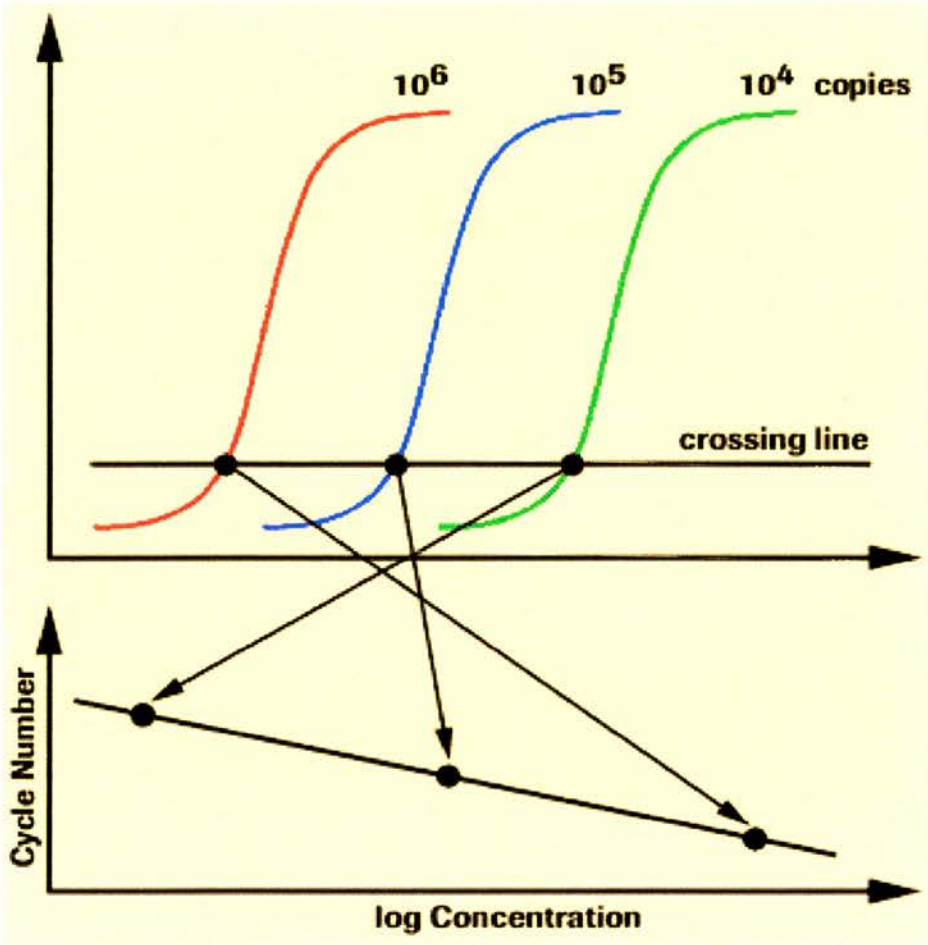


Figure 28: The principle of real-time quantitative RT-PCR: The crossing point values obtained for a set of standards are converted into log concentration values which are directly proportional to the starting number of DNA copies (from Light Cycler™, Roche Diagnostics).

The Light Cycler™ real-time analysis also evaluates the specificity of the amplified product through a melting curve analysis. Since the melting temperature of a DNA product is characteristic of its GC content, length and sequence, melting temperature is used as a form of product identification. The aim following amplification is to observe a single, distinct peak with a high melting temperature. However, a smaller distinct peak with a lower melting temperature is often seen which is usually caused by primer-dimer- or other non-specific product formation.

3.2.5.2 Quantitative real-time PCR

The initial stage of real-time PCR optimisation was similar to that of conventional PCR. Different $MgCl_2$ concentrations (2mM, 3mM, 4mM and 5mM respectively) were tested on four different serial dilutions of the standards. As the real-time reaction consumes more $MgCl_2$ than a conventional thermal cycler, a concentration of 3mM $MgCl_2$ was used in the master mix. The basic components of the real-time PCR reaction using LightCycler™-FastStart DNA Master SYBR Green I kit (Roche Diagnostics) were as follows:

2µl LightCycler™-FastStart DNA Master SYBR Green I master mix containing FastStart Taq DNA polymerase, reaction buffer, dNTP mix (200µM for each nucleotide, dTTP substituted for dUTP), SYBR Green I dye and 10mM $MgCl_2$. Additionally 3.0mM $MgCl_2$ (1.6µl) was added as well as 10pmol (0.5µl) of left and right *c-kit* and *β-actin* internal primers respectively, sterile water (11.6µl) and 2µl of sample cDNA giving a total sample volume of 20µl. FastStart Taq DNA polymerase is a modified form of

thermo-stable recombinant Taq DNA polymerase. It is inactive at room temperature but "activated" at higher temperatures which minimizes non-specific amplification.

Care was taken not to touch the sides of the glass capillaries during sample preparation as this could interfere with the signal reading.

As the thermal cycler used for this experiment (Light Cycler™, Roche Diagnostics) differs significantly from that of a standard instrument, manufacturer's advice regarding cycle conditions for the given product sizes was sought and the following programmes chosen:

An initial pre-denaturation step of 10 minutes at 95°C was followed by 45 cycles of denaturation for 10 seconds at 95°C, annealing for 5 seconds at 57°C (55°C for *β-actin*), extension for 10 seconds at 72°C. This was followed by the melting curve analysis consisting of three segments where following an increase in target temperature to 95°C, it was lowered to 65°C for 15 seconds with a temperature transition rate of 20°C/second. In the final segment the target temperature was again increased to 95°C before the final cooling cycle at 40°C for 30 seconds. In order to ensure that only a single product had been amplified, the glass capillaries were spun down following completion of the cycle and the PCR products run out on an agarose gel.

Following optimisation, all serial dilutions of standards were run in duplicate. This was carried out in order to ensure repeatability of the reaction as well as to provide the

standard curve as described. Following this, the clinical samples were run along with three serial dilution samples in order to provide the reference upon which to adjust sample values to the standard curve. A maximum of 32 samples could be run in each batch. A negative control and three references from the standard curve dilution series were always included with each run. This meant that small intestine normal and colic samples were run separately from the large intestine normal and colic samples. As the annealing temperature differed between the two genes, the c-*kit* and *β-actin* were also run separately.

3.2.6 Sample assessment and statistical analysis

All sequence results files were processed and contigs assembled using the Staden Package version 2002.0 (Staden *et al.* 2000). Sequences were aligned using the EMMA program (interface for ClustalW) within the Human Genome Mapping Project (HGMP) Jemboss package. The resulting multiple sequence alignment was exported into the Molecular Evolutionary Genetics Analysis program (MEGA2.1) to produce the figures used within this thesis.

Analysis of real-time RT-PCR data included the actual quantification values, the specificity control of the reaction using melting curve analysis, and the comparison between the real-time and immunohistochemical quantitative data.

The real-time quantitative data were obtained by calculating the mean *β-actin*-values (A) for the control animals in each of the two groups (small and large intestine). This mean value was divided by the individual animal's *β-actin*-value (B). This value (C) was divided by the individual animal's c-*kit*-value (D) to produce the normalised c-*kit*-value (E); (B/A=C; D/C=E).

The specificity of the reactions was evaluated on the illustrated melting curve analysis. One sample, 03/209, was found to have a non-specific melting curve on one analysis, but this was repeated as it was suspected to be due to a pippeting error. Repeat evaluation demonstrated a normal melting curve and the sample was subsequently included in the study.

As neither the real-time nor the immunohistochemical data were normally distributed, Mann-Whitney statistical analyses were performed. A significance level of $p<0.05$ was assumed.

3.3 Results

3.3.1 Amplification and sequence confirmation of c-*kit* and *β-actin* by RT-PCR

c-*kit* and *β-actin* were amplified by RT-PCR as described in section *3.2.4.2.* Amplified products were run on agarose gels to confirm the product size (Figures 29-31). PCR products were gel extracted for sequencing and BLAST searches of the sequence data

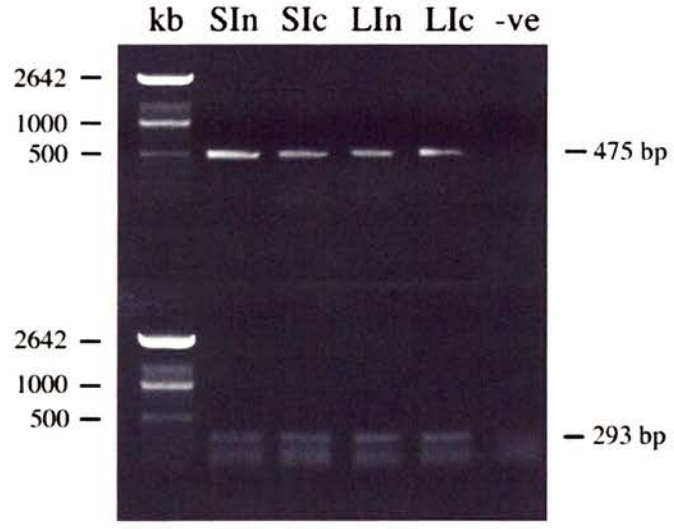


Figure 29: Amplified external (top row) and internal (bottom row) products of c-kit amplification for all four horse groups. SIn: small intestine sample normal; SIc: small intestine sample colic (diseased); LIn: large intestine sample normal; LIn: large intestine sample colic (diseased).

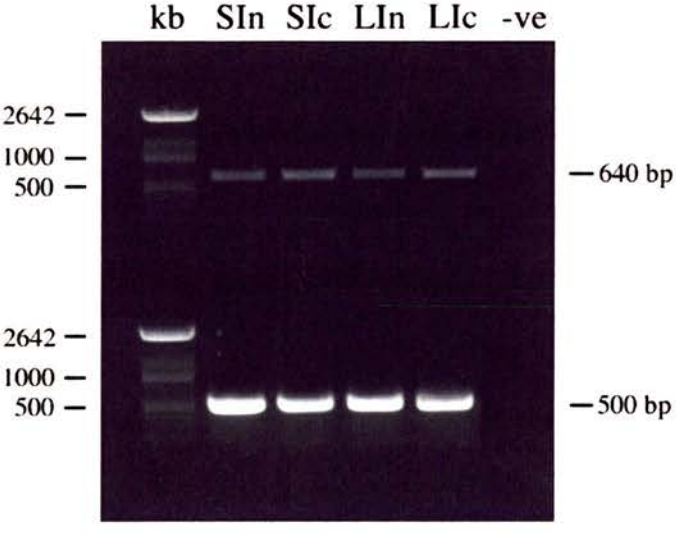


Figure 30: Amplified external (top row) and internal (bottom row) products of β-actin amplification for all four horse groups. SIn: small intestine sample normal; SIc: small intestine sample colic (diseased); LIn: large intestine sample normal; LIn: large intestine sample colic (diseased).

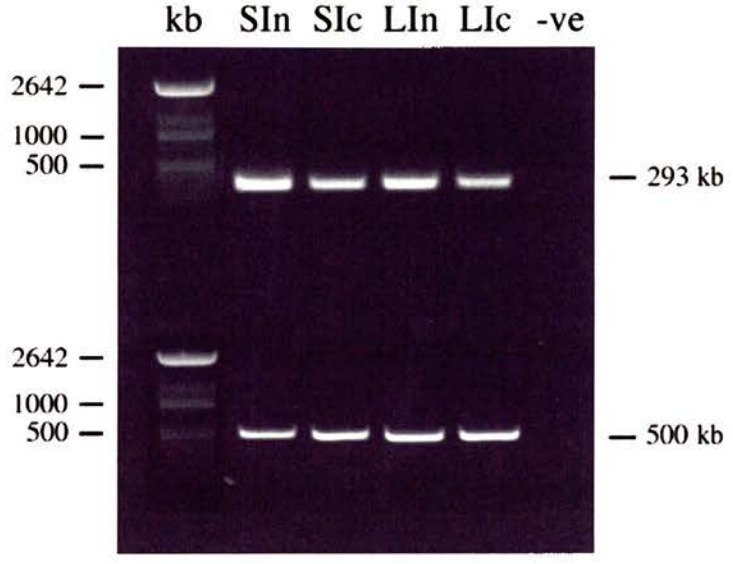


Figure 31: Amplified nested products of c-kit (top row) and β-actin (bottom row) amplification for all four horse groups. SIn: small intestine sample normal; SIc: small intestine sample colic (diseased); LIn: large intestine sample normal; LIn: large intestine sample colic (diseased).

confirmed that the amplified products corresponded to c-*kit* and *β-actin* respectively. This result allowed further preparation and subsequent analysis of the quantitative real-time RT-PCR data.

3.3.2 Real-time PCR data analysis

Analysis of the real-time PCR was performed using the Light Cycler™ (Roche) software analysis programme. These data are illustrated in Appendix 4 and demonstrate the amplification of c-*kit* and *β-actin* for the two groups together with the crossing-point values. The melting curve data are also illustrated in Appendix 4. These demonstrate a single melting peak for *β-actin*, although for c-*kit* primer-dimer formation is evident by the presence of a smaller melting peak. These samples were spun down and run on a gel to confirm that this smaller melting peak was indeed primer-dimer rather than a separate, distinct product.

All measured data for c-*kit* and *β-actin* are described in Table 10. The ranges of these data, as well as the normalised c-*kit* values for all horse groups are further described in Figures 32-34. As seen from Figures 32 and 33, there were outlying values for both c-*kit* and *β-actin* in both the small and large intestine group. However, when the c-*kit* values were normalised, these values were adjusted although one outlier value was still present in the small intestine normal group (Figure 34). There was no particular reason why this animal (01/488) should have a value that was outside the remainder of the group. This animal was the very first sample to be collected for this investigation and was stored for

Table 10: Measured *β-actin* and c-*kit* real-time RT-PCR data as well as calculated, normalised c-*kit* values for all horses included in study.

Horse group	Measured *β-actin* values	Measured c-*kit* values	Normalised c-*kit* value
Small intestine normal			
SSPCA	0.00003651	0.00000001976	0.000000052
03/488	0.00006524	0.00000002504	0.000000038
01/488	0.000000000013	0.00000007379	0.56
02/252	0.000000000322	0.00000001735	0.00527
02/56	0.000000000340	0.00000001190	0.00323
Lamb	0.0002594	0.00000010120	0.0000000389
01/462	0.0004034	0.00000009524	0.0000000235
03/381	0.00001218	0.00000005156	0.0000004187
Small intestine colic			
00/656	0.00003793	0.00000001614	0.0000000421
02/90	0.0003639	0.00000008538	0.0000000233
09/06/03	0.0001409	0.00000002657	0.0000000184
03/435	0.0003480	0.00000005641	0.0000000160
02/110	0.0002018	0.00000009552	0.0000000470
02/97	0.0009640	0.00000031010	0.0000000321
03/209	0.00001037	0.000000000055	0.0000000048
27870	0.00001910	0.00000001126	0.0000000575
Large intestine normal			
Wispa	0.0002289	0.00000002962	0.0000000307
99/788	0.0001042	0.00000001502	0.0000000349
03/488	0.0001983	0.00000001648	0.0000000196
03/303	0.0004335	0.00000002357	0.0000000128
03/381	0.0001388	0.00000004987	0.0000000857
Wobbler	0.0004748	0.00000008286	0.0000000417
03/124	0.0001286	0.00000002391	0.0000000434
Large intestine colic			
03/242	0.001792	0.00000006205	0.0000000084
27644	0.0001052	0.00000004068	0.0000000924
03/340	0.0002088	0.00000010070	0.0000001163
18/05/03	0.00001962	0.00000000283	0.0000000247
Pedrita	0.0002659	0.00000010950	0.0000000996
28570	0.0003571	0.00000069480	0.0000004722
28535	0.001528	0.00000001977	0.0000000030
Annie B	0.000000013	0.00000000141	0.00002
28225	0.00005	0.00000000728	0.0000000340
28287	0.000000011	0.00000000302	0.0000075

a period of time prior to processing. However, the RNA quality had been checked prior to further processing and so was unlikely to be a contributing factor. It is possible that this value is still within the normal range. For this reason, all data were included in the statistical analysis. This demonstrated no significant difference in the c-*kit* transcription levels between normal and diseased small intestinal samples (p=0.9581) or between the normal and diseased large intestinal samples (p=0.4350).

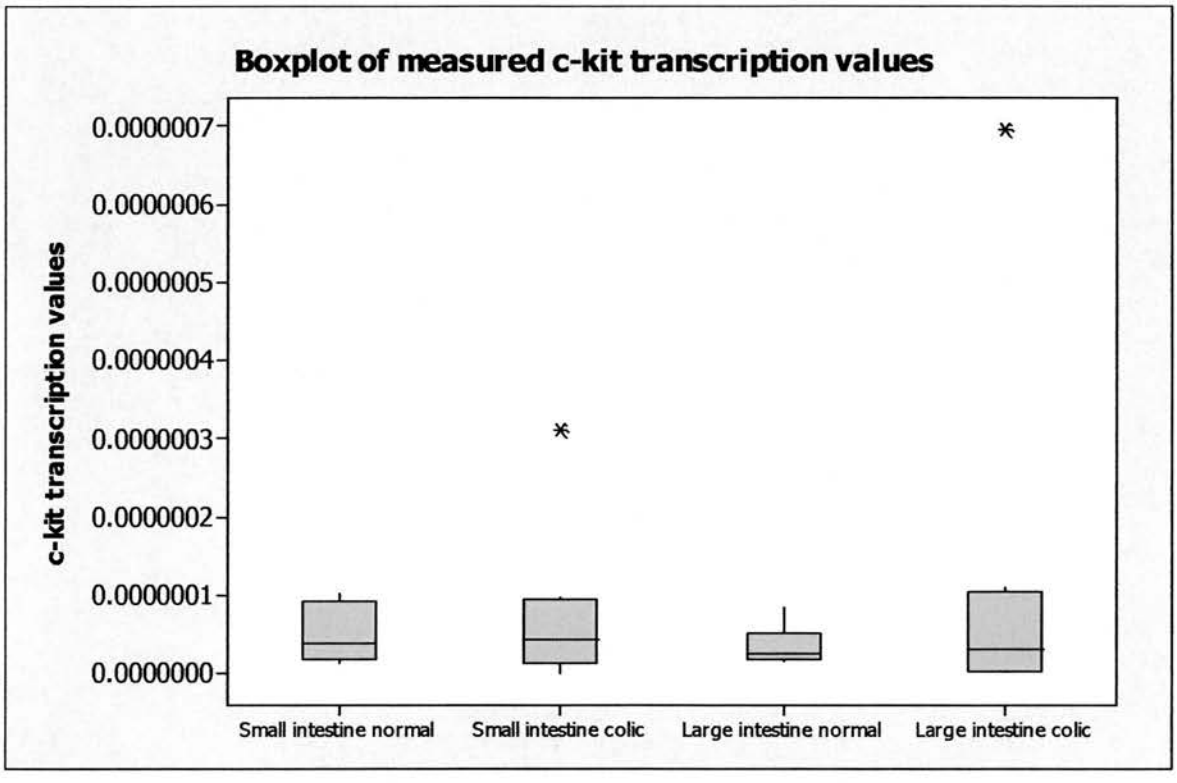


Figure 32: Boxplot of measured c-*kit* transcription values. The ranges of the relative c-*kit* transcription values obtained for each horse group are illustrated. These data are also described in Table 10. Outlier values were observed in the small and large intestine colic groups (star). Small intestine normal (n=8), small intestine colic (n=8), large intestine normal (n=7), large intestine colic (10).

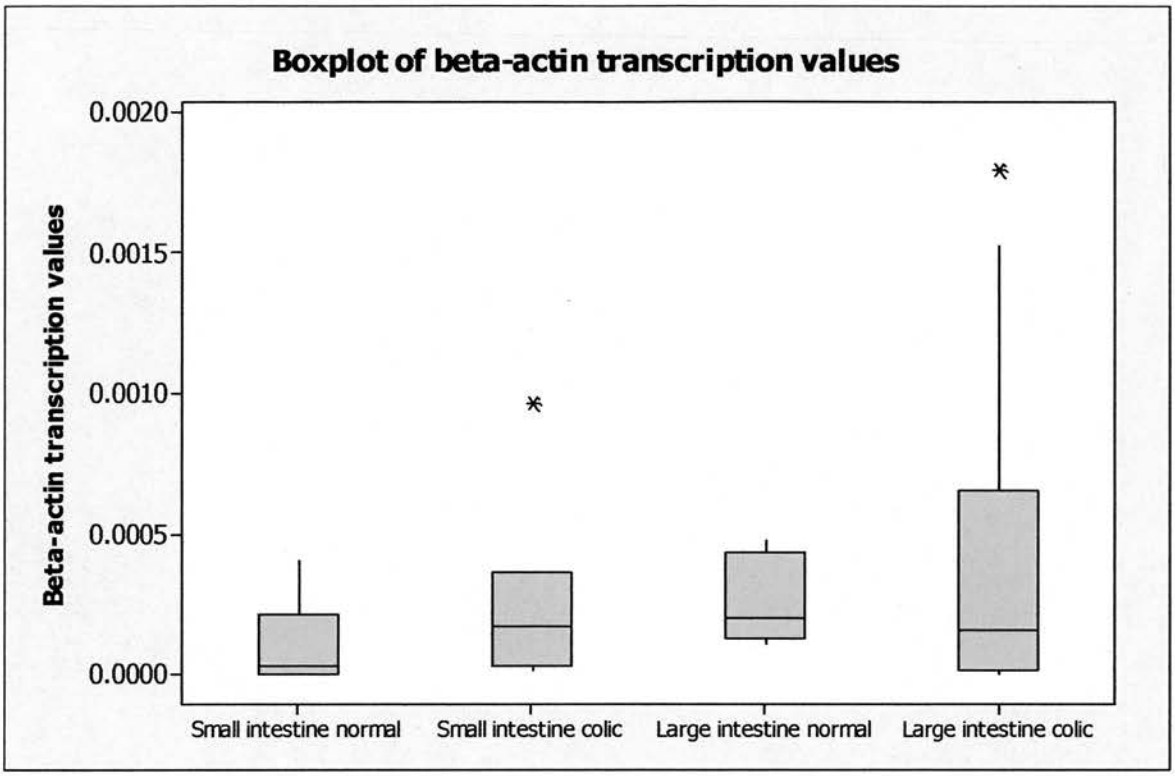


Figure 33: Boxplot of measured *β-actin* transcription values. The ranges of the relative *β-actin* transcription values obtained for each horse group are illustrated. These data are also described in Table 10. Outlier values were observed in the small and large intestine colic groups (star). Small intestine normal (n=8), small intestine colic (n=8), large intestine normal (n=7), large intestine colic (10).

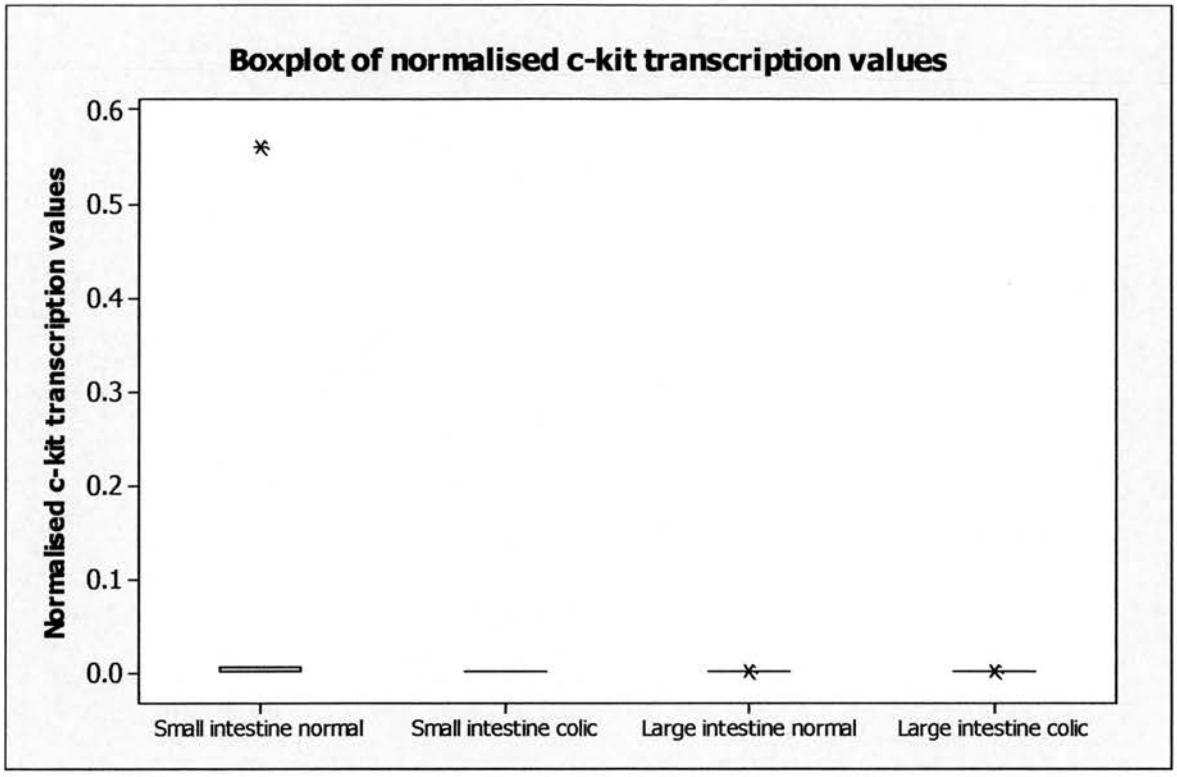


Figure 34: Boxplot of normalised c-*kit* transcription values where the measured c-*kit* values were normalised to the measured values of the control gene *β-actin*. These data are also described in Table 10. Outlier values were observed in both normal small intestine and normal and colic large intestine colic groups (star) (Table 10). However, because of the narrow ranges of the latter two groups created by 2 larger outliers in the small intestine normal group, the outliers in the normal and colic large intestine colic groups appear to lie within the estimated ranges.

Small intestine normal (n=8), small intestine colic (n=8), large intestine normal (n=7), large intestine colic (10).

3.3.3 Immunohistochemical grading

Tissue sections were labelled by c-Kit immunohistochemistry to detect and quantify c-Kit protein immunoreactivity as described in detail in the previous chapter. The density of c-Kit-immunoreactive ICC was compared between cases and compared to c-*kit* transcription levels.

The individual animals' ICC density grades are described in Table 11. There was no statistical difference in the degree of immunoreactivity in the myenteric plexus region (MP) or of the circular muscle region (CM) ($p=0.2976$ and $p=0.1832$ respectively) between the normal and colic small intestinal samples. The percentage of grades allocated to the different anatomical areas is illustrated in Figure 35 and 36. As for the small intestine, there was no significant difference in the degree of immunoreactivity in the MP region between the normal and colic large intestinal samples ($p=0.0669$). However, there was a significant reduction in the degree of immunoreactivity in the CM between the normal and colic large intestinal samples ($p=0.0428$). The percentage of grades allocated to the MP and CM regions of the large intestine is illustrated in Figure 37 and 38. The combined MP and CM grades for each group demonstrated no significant statistical difference between normal and diseased small intestinal immunoreactivity ($p=0.2716$), whereas there was a significant difference between normal and diseased large intestinal grades ($p=0.0248$). The distribution of grades allocated to the different anatomical areas for the four horse groups is illustrated in Figures 39 and 40. This demonstrated a wider range of grades allocated to animals in the

Table 11: Immunohistochemical consensus data for all horses included in the study. The allocated density grades range from 0-3 where absent=0, sparse=1, moderate=2 and abundant=3.

Horse group	Myenteric plexus region (MP)	Circular muscle (CM)	Combined values (MP and CM)
Small intestine normal			
SSPCA	2	1	3
03/488	2	2	4
01/488	2	2	4
02/252	2	1	3
02/56	2	1	3
Lamb	2	1	3
01/462	2	1	3
03/381	2	1	3
Small intestine colic			
00/656	2	1	3
02/90	1	0	1
09/06/03	2	2	4
03/435	2	1	3
02/110	0	0	0
02/97	2	1	3
03/209	0	0	0
27870	Discarded	Discarded	Discarded
Large intestine normal			
Wispa	2	3	5
99/788	2	2	4
03/488	2	3	5
03/303	2	3	5
03/381	2	2	4
Wobbler	2	2	4
03/124	2	3	5
Large intestine colic			
03/242	0	0	0
27644	2	2	4
03/340	0	0	0
18/05/03	1	2	3
Pedrita	2	3	5
28570	1	1	2
28535	1	1	2
Annie B	2	2	4
28225	1	2	3
28287	2	2	4

colic group (both small in large intestine) in the myenteric plexus region compared to the control groups. A similar observation was made for the grades allocated to the circular muscle layer, with the widest range being present in the large intestine colic group.

H&E examination of the tissue samples indicated that one diseased small intestinal sample (03/381) was not of sufficient quality to be included in the immunohistochemical study. This reduced quality was caused by inappropriate fixation of the sample. However, it should be emphasised that the parallel RNA quality assessment found this sample to be of good quality.

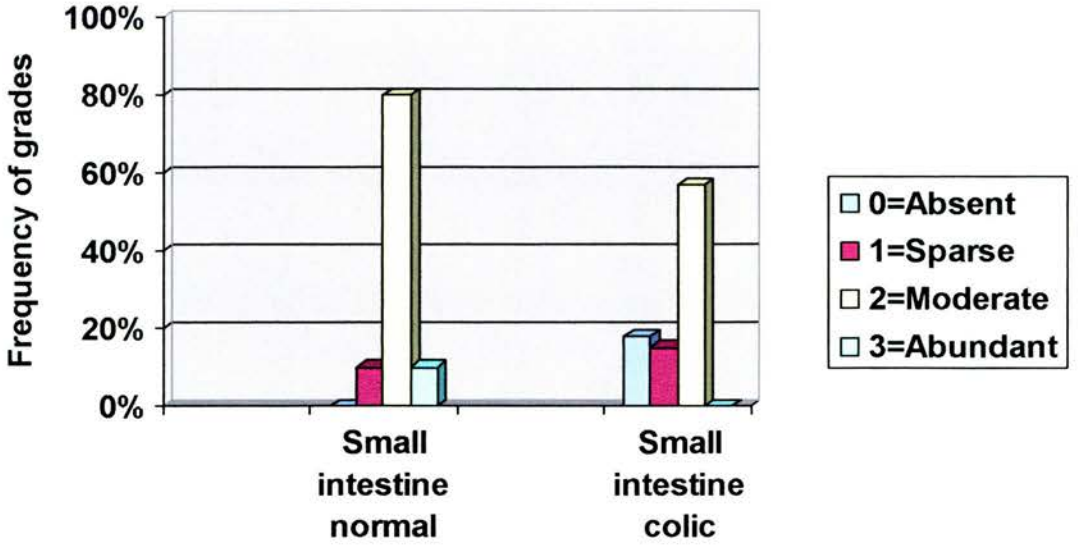


Figure 35: ICC density in the myenteric plexus region in the small intestine of normal horses (n=8) and horses with a small intestinal obstructive lesion (n=8). Frequency grade (%) represents the proportion of specimens that were assigned to a particular density grade: absent (0), sparse (1), moderate (2) and abundant (3).

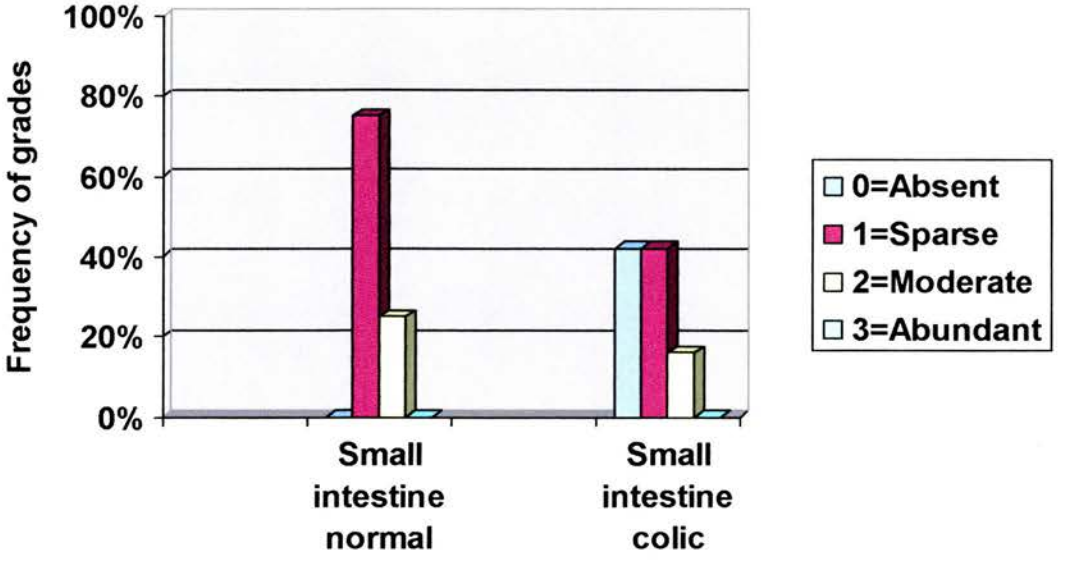


Figure 36: ICC density in the circular muscle region in the small intestine of normal horses (n=8) and horses with a small intestinal obstructive lesion (n=8). Frequency grade (%) represents the proportion of specimens that were assigned to a particular density grade: absent (0), sparse (1), moderate (2) and abundant (3).

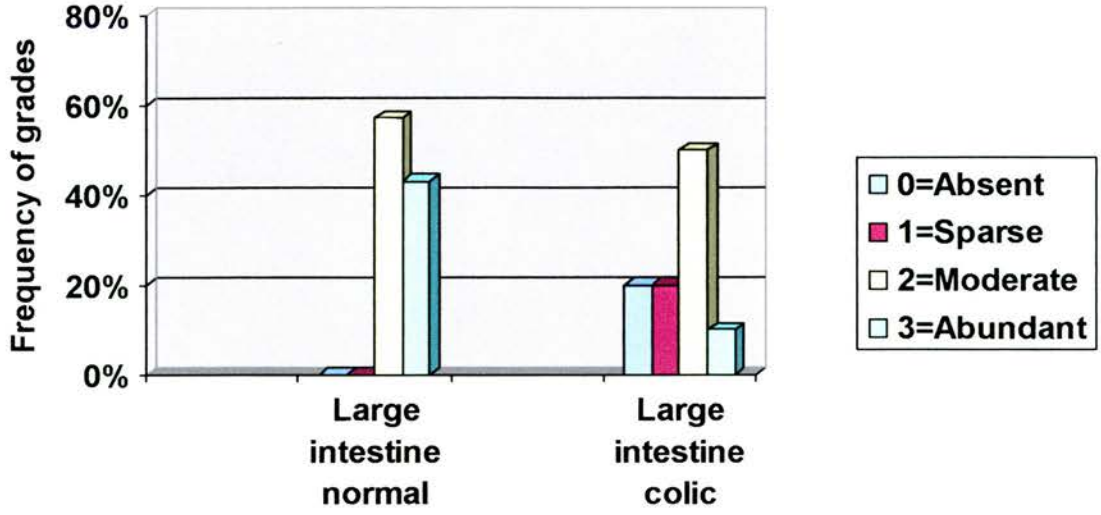


Figure 37: ICC density in the myenteric plexus region in the large intestine of normal horses (n=7) and horses with a large intestinal obstructive lesion (n=10). Frequency grade (%) represents the proportion of specimens that were assigned to a particular density grade: absent (0), sparse (1), moderate (2) and abundant (3).

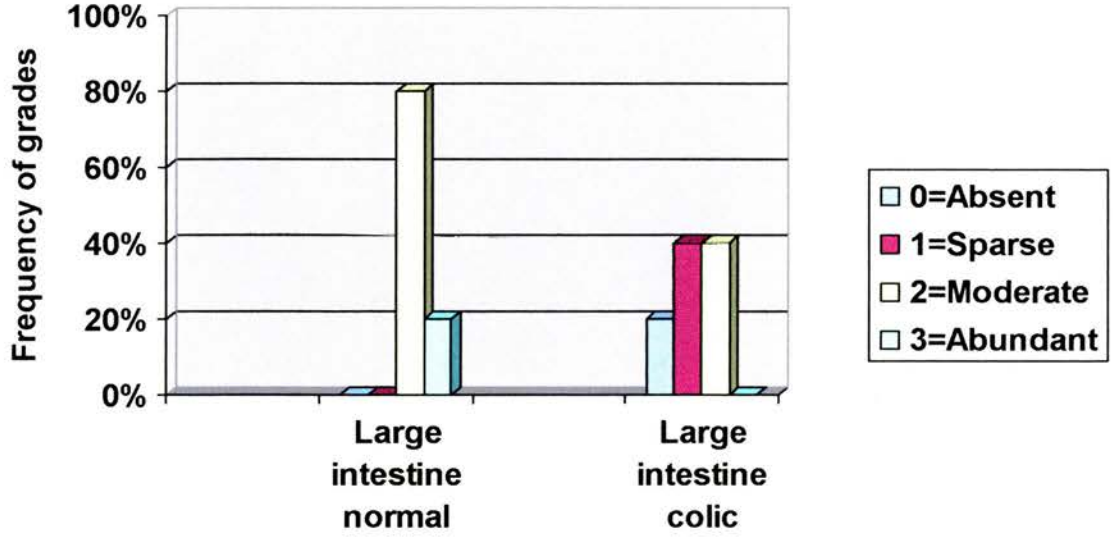


Figure 38: ICC density in the circular muscle region in the large intestine of normal horses (n=7) and horses with a large intestinal obstructive lesion (n=10). Frequency grade (%) represents the proportion of specimens that were assigned to a particular density grade: absent (0), sparse (1), moderate (2) and abundant (3).

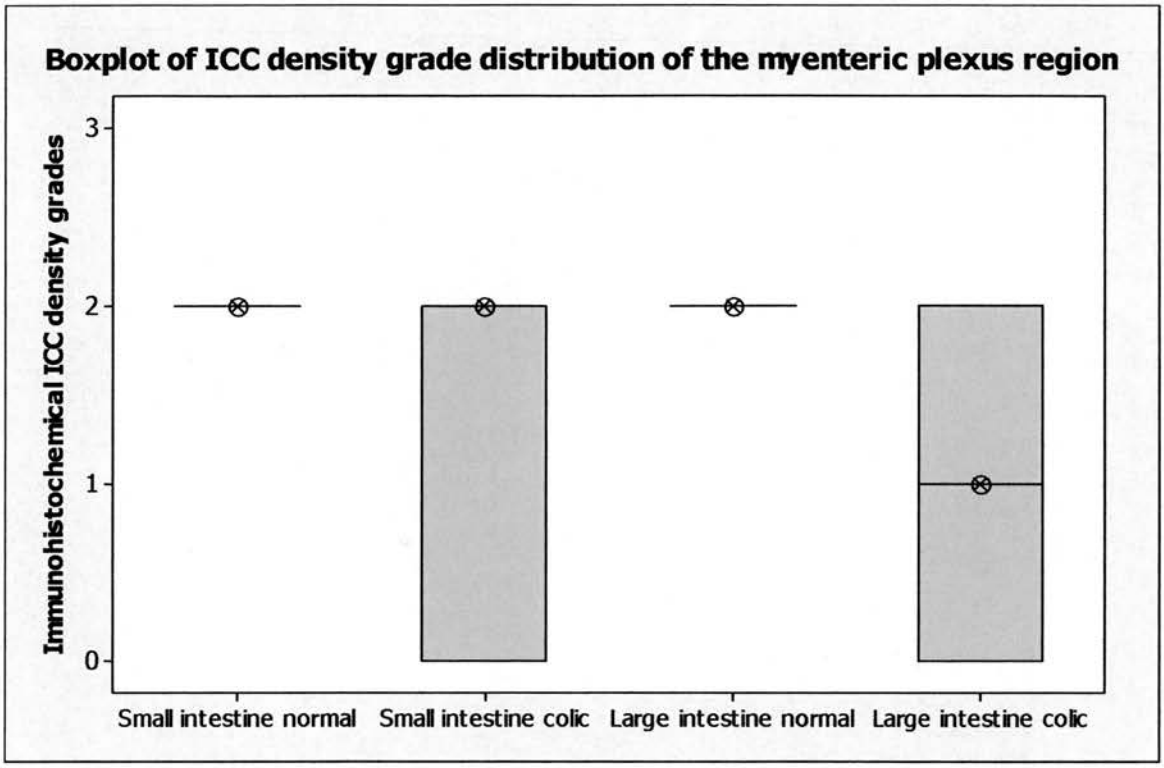


Figure 39: Boxplot of immunohistochemical ICC density grades. The myenteric plexus region of all tissue samples in all four horse groups were assessed and allocated a grade based on the density of ICC immunoreactivity present. Absent immunoreactivity was given a grade of 0, sparse =1, moderate =2 and abundant immunoreactivity a grade of 3. The symbol mark (*) present in each column signifies the median ICC density value for that particular group.

Small intestine normal (n=8), small intestine colic (n=7), large intestine normal (n=7), large intestine colic (n=10).

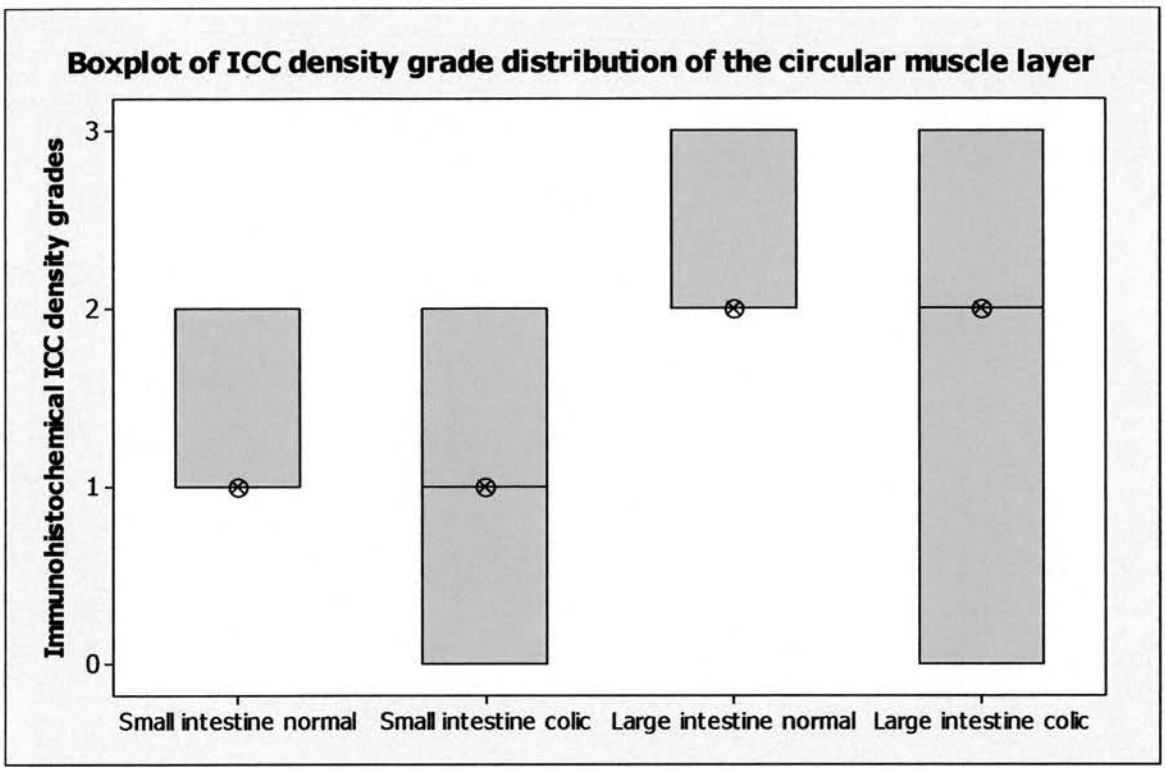


Figure 40: Boxplot of immunohistochemical ICC density grades. The circular muscle layer of all tissue samples in all four horse groups were assessed and allocated a grade based on the density of ICC immunoreactivity present. Absent immunoreactivity was given a grade of 0, sparse =1, moderate =2 and abundant immunoreactivity a grade of 3. The symbol mark (*) present in each column signifies the median ICC density value for that particular group.

Small intestine normal (n=8), small intestine colic (n=7), large intestine normal (n=7), large intestine colic (n=10).

3.4 Discussion

This is the first clinical study to investigate c-*kit* gene transcription levels in relation to the degree of immunoreactivity of the protein product it encodes, c-Kit.

Mammalian gene expression is regulated primarily at the level of initiation of transcription (Beyersmann 2000). Therefore, it was expected that the reduction in c-Kit immunoreactivity would be a reflection of altered c-*kit* mRNA transcription levels. Surprisingly, this study demonstrated a significant reduction in c-Kit immunoreactivity in horses with an obstructive disorder of the large intestine, although no changes in the c-*kit* gene transcription levels were observed in any of the study groups. The strength of these findings is supported by the high level of accuracy and reproducibility real-time RT-PCR method offers (Heid *et al.* 1996). The discrepancy between c-*kit* mRNA (transcription) levels and immunoreactivity was an intriguing result for which there are a number of possible explanations.

It is possible that changes to transcription levels did take place, but that this occurred prior to sample collection and levels subsequently returned to normal. The time interval between horse owners observing the first clinical signs of abdominal pain and the horse undergoing corrective surgery varied with the individual cases, but this could often be as long as 6-8 hours. One study using Northern blot analysis to investigate c-*kit* expression in human endothelial cells demonstrated a reduction in mRNA levels following exposure to an inflammatory cytokine (IL-1α) (König *et al.* 1997). This downregulation occurred

within 2 hours of stimulation and c-*kit* mRNA was almost undetectable after 24 hours (König *et al.* 1997). If we can apply the findings from this study to the current investigation, it seems reasonable to assume that if changes in transcription levels did occur, it would still be detectable in the given time frame, especially in the presence of a continuing pathogenic stimulus and environment. In an ideal situation, sample collection would have taken place soon after the onset of the intestinal insult. However, in a clinical setting, this is often not possible as the majority of horses have to travel a distance to a referral hospital for surgical treatment, making a delay inevitable.

As another explanation, it is possible that important regulation of protein production occurs at a post-transcriptional level and that a pool of mRNA exists. Protein synthesis represents another key control point for gene regulation (Dever 2002). This enables cells to respond rapidly to changes in the extracellular environment by producing abundant amounts of specific proteins from a stored pool of mRNA (Dever 2002). Conversely, in response to starvation and stress a reduction in the rate of protein synthesis occurs, which is mediated by the phosphorylation of translation initiation factor-2 (eIF-2) (Hinnebusch 1994). This downregulation of protein synthesis limits cell division under adverse growth conditions which may protect against the deleterious effects of toxic agents and provides a means of conserving resources that are needed for survival (Hinnebusch 1994; Sheikh and Fornace Jr. 1999). Currently, there is no specific information available on translational control of the c-Kit receptor, although regulation mechanisms of proteins encoded by other proto-oncogenes have been proposed (Willis 1999). This includes structured regions which inhibit scanning mechanisms of

translation, regulatory upstream open reading frames and internal ribosome entry segments which are capable of initiating cap-independent translation (Willis 1999). It seems reasonable to hypothesise that similar translational regulatory responses to cellular stress also applies to these and indeed the c-Kit receptor.

It is also possible that the reduced c-Kit immunoreactivity was caused by acute injury to the c-Kit receptor exceeding the rate of protein synthesis thereby resulting in an overall reduction in c-Kit immunoreactivity. As discussed in the earlier chapter, previous studies have demonstrated ultrastructural changes to ICC during experimentally created inflammatory conditions (Lu *et al.* 1997; Der *et al.* 2000), although another study failed to demonstrate a concomitant reduction in c-Kit immunoreactivity under these conditions (Wang *et al.* 2002).

The presence of protease-like enzymes in the local environment could not only participate in receptor inactivation but also participate in the destruction of receptor agonists such as the natural ligand of c-Kit, stem cell factor (SCF) (Huang *et al.* 1990; Williams *et al.* 1990; Zsebo *et al.* 1990; Ossovskaya and Bunnett 2004). Studies in the mouse with a mutation of the Steel (Sl) locus on chromosome 10, have provided support that the c-Kit signalling pathway is necessary for proper development of pacemaker-generating ICC networks (Ward *et al.* 1995). Equally, SCF is also important in the maintenance of this tyrosine kinase receptor in adulthood (Wu *et al.* 2000). Local presentation of SCF increases *in vitro* expression of c-Kit immunoreactive ICC (Rich *et al.* 2003). In other words, it is possible that decreased levels of SCF presented to the c-

Kit receptor, as well as injury to the receptor itself, may have contributed to the reduction in c-Kit immunoreactivity observed in some horses in the current study. This is an area that warrants further investigation.

It is of course possible that the discrepancy between transcription levels was related to the relatively low power of this study to detect differences due to the small sample size. It is possible that an undetected small to moderate reduction in c-*kit* transcription levels resulted in a significant decrease in c-Kit immunoreactivity. The small sample size could also explain why the differences in small intestinal c-Kit immunoreactivity were non-significant.

The findings in this study also need to be put into a clinical context. As discussed in the previous chapter, two main questions arise. Firstly, is the reduction in ICC density related to the motility disturbance of a particular animal and secondly, were these changes already present in horses with a history of previous colic episodes again returning to the cause and effect issue. In other words, did the reduction in c-Kit immunoreactivity occur in association with the current intestinal insult or could it have happened during a previous colic episode resulting in disrupted ICC networks and thereby making these animals more prone to developing colic?

Several experimental studies in other mammalian species have demonstrated that changes to ICC densities and networks are associated with altered slow wave activity and thereby intestinal motility patterns (Ward *et al.* 1994; Huizinga *et al.* 1995;

Torihashi *et al.* 1995; Kenny *et al.* 1998b; Mikkelsen *et al.* 1998; Torihashi *et al.* 1999). In view of these findings, it seems reasonable to assume that disrupted ICC networks have similar effects on motility patterns also in the horse. Furthermore, this investigation as well as the larger immunohistochemical study in the previous chapter demonstrated that changes to ICC densities do occur in a number of horses with motility disorders and so it seems unlikely that these changes are purely coincidental without any functional and thereby clinical relevance.

The results confirm the observations made in the previous chapter that a proportion of animals do suffer from repeated colic episodes and, in the absence of any obvious physical cause, it is reasonable to assume that some of these horses may indeed suffer from an underlying intrinsic motility dysfunction. In the current study, three out of 10 horses in the large colon group had a history of previous colic episodes but because of the relatively small sample number it was not possible to carry out meaningful statistical analysis of these data. However, it was noted that only one of these three animals had reduced ICC densities from which it is possible to draw three conclusions. Firstly, ICC may not be important in equine intestinal motility disorders although bearing in mind the previous discussion, this seems unlikely. Secondly, it is possible that focal reductions in ICC densities were present in the two cases with apparently normal ICC densities but that that the changes did not involve the particular area examined. This is a possibility considering the size of the equine large colon but one that will be very difficult to demonstrate without obtaining further tissue samples. Finally, it is of course possible that the intestinal disorders of these two horses were not related to an intrinsic motility

problem but rather caused by other factors such as management and feeding practices as discussed in detail in the introduction of this thesis. For these two particular horses, detailed information on management was not obtainable and hence this possibility remains a speculation.

Regardless of these two horses, one animal with recurrent colic episodes did have reduced ICC densities so the question of cause and effect discussed in some detail in the previous chapter is still relevant. The current study did not detect any transcriptional changes to the c-*kit* mRNA levels in any of the horse groups including this particular animal. From this it is reasonable to hypothesise that the c-*kit* gene transcription levels do not change in horses with recurrent colic episodes and other avenues should be explored.

Control measurements form a fundamental part of all quantitative procedures. In the current study, the quantity of the target gene, c-*kit*, was compared to the constitutively expressed *β-actin*. This allowed the assessment of differences in sample loading and reaction efficiencies, thus providing a means to evaluate and subsequently adjust for intrinsic experimental variations (Stürzenbaum and Kille 2001). The selection of a suitable control gene has been subject to much discussion as there are advantages and limitations with the majority of these. The chosen housekeeping gene for this study, *β-actin*, is one of the major components of cytoplasmic microfilaments in eukaryotic cells (Stürzenbaum and Kille 2001). Although it is one of the most widely used internal

controls, there is now some evidence to suggest that the concentration of this gene can vary with muscle type, development, cell culture conditions, pathologically and potentially between cells within tissues (Drew and Murphy 1997). However, there is currently only a limited choice of alternative housekeeping genes available. Another commonly used gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), was not chosen as it has been reported that its use should be avoided if hypoxic conditions existed (Zhong and Simons 1999). As it was possible that a proportion of clinical samples would have experienced some degree of hypoxia, this gene was considered unsuitable. Other now more commonly used controls are ribosomal subunits (Stürzenbaum and Kille 2001). Unfortunately, this was not an option in the current investigation as the ribosomal subunits are not polyadenylated (Stürzenbaum and Kille 2001), and therefore cannot be exploited as the cDNA used in the current study was derived from total RNA utilising poly T-primers (oligo-dT primers) in the RT reaction. In view of this current information and choice of control genes available, *β-actin* was considered the most suitable although there are clearly some limitations to its suitability in this role that could have influenced the normalised c-*kit* values.

The results of this study indicate that future investigations into the reduction in c-Kit immunoreactivity should focus on post-transcriptional c-*kit* regulation as well as on changes that occur to the c-Kit protein under periods of cellular stress. This includes quantitation of the c-Kit protein through Western blot analysis to get an accurate estimate of the protein level both in normal and diseased animals, including those with

recurring colic episodes. This data should ideally be looked at in parallel with immunohistochemical and ultrastructural studies. Furthermore, parallel intracellular electrophysiological recordings would obtain valuable functional information. As an essential part of this process, the normal electrophysiological activity of the equine large colon needs to be characterised prior to evaluating diseased tissue. This is addressed in the following chapter.

4 ELECTROPHYSIOLOGICAL STUDIES

4.1 Introduction and aims

The previous studies in this thesis have demonstrated a significant reduction in ICC densities in horses with an obstructive disorder of the large intestine and it was hypothesised that these changes may be associated with intestinal motility dysfunction in at least some of these animals. To further investigate this hypothesis, functional information on the effect these changes may have on electrical activity in the equine large colon was considered. The majority of studies investigating motility patterns in the equine large colon have been extracellular myoelectrical studies which require surgical implantation of electrodes into the bowel wall (Sellers *et al.* 1979; Ruckebusch and Fioramonti 1980; Ruckebusch 1981; Roger and Ruckebusch 1987; Ruckebusch and Roger 1988; Merritt *et al.* 1995; Roussel *et al.* 2000). These studies have revealed a complex myoelectrical pattern that still warrants further investigation in order to obtain a more complete picture of the activity in this region. Intracellular recording techniques have been performed in the small intestine of the horse (Rakestraw *et al.* 2000; Hudson *et al.* 2001c, 2002). Hudson *et al.* (2001c, 2002) were able to demonstrate slow wave activity in the ileum of both normal animals as well as horses with EGS using this technique. Currently, slow wave activity has not been recorded in the equine large colon using this recording technique although it has been described in several other mammalian species including man, dog, cat and rat (Christensen *et al.* 1969; Durdle *et al.* 1983; Smith *et al.* 1987a, b; Rae *et al.* 1998; Plujà *et al.* 2001). These studies have demonstrated a complex electrical pattern which may involve two separate pacemaker

centres located at the submucosal border of the circular muscle layer as well as in the myenteric plexus region (Smith *et al.* 1987b; Plujà *et al.* 2001).

The aim of this study was to characterise the *in vitro* electrical activity of the equine pelvic flexure region in order to provide a basis for the interpretation of samples collected from diseased animals. The pelvic flexure was chosen as this anatomical area is thought to be an important motility control centre in the horse and is also important clinically as many equine motility disorders, such as impactions, involve this region (Lowe *et al.* 1980; Sellers *et al.* 1982a, 1984; White 1990; Lopes and Pfeiffer 2000).

4.2 Materials and methods

Intestinal samples including pelvic flexure apex (located at the junction between the left ventral colon and left dorsal colon) as well as a segment of ileum (at a position level with the midpoint of the ileocaecal fold) were collected immediately (within 30 minutes) following euthanasia by intravenous administration of quinalbarbitone sodium BP (400mg/ml) /cinchocaine hydrochloride BP (25mg/ml) (Arnolds) from 7 adult horses. The details of all horses are described in Table 12. In 1 animal (04/286), only pelvic flexure was collected and in 1 horse (04/392), jejunum rather than ileum was collected. Three horses were euthanised for conditions not relating to the GI tract, while 3 horses had EGS and 1 horse was euthanised because of an inflammatory condition restricted to the proximal small intestine. The ages of the animals included in the study ranged from

5-20 years (median 9.0 years, mean 10.0 years) and comprised 6 geldings and 1 mare (Table 12).

Table 12: Details on horses included in electrophysiological study

Horse	Age (Yrs)	Sex	Sample collected	Reason for euthanasia
04/219	9	G	IL/PF	CNS disease
04/286	20	F	PF	Orthopaedic disease
04/569	9	G	IL/PF	Guttural pouch mycosis
00/345	6	G	IL/PF	Anterior enteritis
02/62	14	G	IL/PF	Acute grass sickness
04/391	7	G	IL/PF	Chronic grass sickness
04/392	5	G	JEJ/PF	Chronic grass sickness

Key: G: Gelding, F: Female, IL: Ileum, PF: Pelvic flexure, JEJ: Jejunum; CNS: Central nervous system

During sample collection, particular care was taken that only the edges of harvested tissue were manipulated with forceps. Once collected, the tissue samples were immediately placed in modified Krebs solution. This consisted of 120.7mM sodium chloride, 5.9mM potassium chloride, 25mM sodium bicarbonate, 1.2mM magnesium sulphate, 2.5mM calcium chloride, 1.5mM sodium dihydrogen phosphate and 11.5mM glucose that had been oxygenated with 95% oxygen and 5% carbon dioxide prior to sample collection in order to provide optimum conditions for tissue viability.

All samples were rinsed in fresh Krebs solution and the segments of small intestine (jejunum and ileum) were opened along the mesenteric border with scissors. The tissue samples were subsequently pinned out on a moistened cork board with the mucosal surface facing down and a 1mm thick cross-section was cut with a double-bladed knife.

In the ileum and jejunum, the orientation of the cut was parallel to the muscle fibres of the longitudinal muscle layer. In the pelvic flexure, samples were cut to allow both parallel and cross-sectional orientations of the circular muscle layer.

For all animals investigated (apart from 04/286), a section of small intestine as well as two sections of pelvic flexure (cross-sectional and parallel orientation of the circular muscle layer) were pinned out on a Sylgard plate. Once pinned out, the mucosa was carefully dissected away from the remainder of the tissue and the preparation superfused with oxygenated Krebs solution which had been warmed prior to reaching the recording chamber. The recording chamber had an approximate volume of 8ml and the rate of superfusion of the warmed, oxygenated Krebs solution was approximately 10ml/minute to ensure that the temperature in the tissue bath was kept between 36.5-37.5°C. The tissue sections were allowed to equilibrate for at least 60 minutes prior to commencing recordings. The actual recording sessions would generally not exceed 8 hours.

Intracellular recordings were made using glass microelectrodes made from either a P97 electrode puller (Sutter Instruments, Navato, CA, USA) or a separate puller designed and made at the Royal (Dick) School of Veterinary Studies, University of Edinburgh, UK. The electrodes were filled with filtered 2M potassium chloride and had resistances ranging from 20-45MΩ. A calibrating graticule in the eyepiece of the dissecting microscope was used to determine the exact position of impaled cells relative to the thickness of the muscle layer. For the circular muscle layer, 0% referred to the muscle at the submucosal border and 100% to that at the myenteric border. For the longitudinal

muscle layer, 0% referred to the muscle at the myenteric border and 100% to that at the serosal border. Recordings from cellular impalements were considered acceptable if there was a sharp initial drop in voltage and if the resting membrane potential (RMP) remained stable. The RMP was measured at the most negative reading for an impalement. Where slow wave activity was recorded, the reading at the "base" of the slow wave was taken as the RMP.

Small intestinal samples were collected in all animals (apart from 04/286) in order to provide a positive control for the subsequent pelvic flexure recordings. All recordings in the small intestinal samples were carried out at a level 10% through the longitudinal muscle layer of the *muscularis externa*, i.e. close to the myenteric plexus region in order to standardise the positive control. Once regular slow wave activity was observed, recordings were carried out on the pelvic flexure samples. All pelvic flexure impalements were made at 5-10% from the submucosal border. Both parallel and cross-sectional samples were included in order to evaluate which orientation gave the best quality of impalements.

Some recordings were performed in the presence of the L-type calcium channel blocker nifedipine (1 μM, Sigma) which was added to the superfusion fluid at an unchanged flow rate.

The data were recorded and stored using an acquisition system (Power Lab 8SP; AD Instruments) interfaced to a Power Macintosh G4 computer. The software package

"Chart" was used to analyse the resting potentials, the amplitude, frequency and duration of membrane potential oscillations and other waveforms.

Due to the small sample size of animals, it was not possible to perform extensive statistical analysis of the data and hence the results are generally qualitative only.

4.3 Results

Slow wave activity was recorded in all small intestinal samples collected (n=6). The frequency of slow waves ranged from 8-12/minute (median 9.0, mean 9.5) between the individual animals although within a particular animal, the frequency remained consistent as demonstrated through repeated cellular impalements. The jejunal sample had the highest slow wave frequency of 12/minute. The mean RMP varied between the different cases (range -12.4- -51.8 mV, median -35.0 mV). The range of the amplitude (not including spike potentials) was 4.0-9.1 mV (median 6.5 mV, mean 6.4 mV) (Table 13). Slow waves were characterised by a fast upstroke and a slower downstroke phase. In the tissue samples where nifedipine had not been added, spikes (action potentials) (2-8mV in amplitude) were occasionally observed superimposed on top of the slow waves (Figure 41). These, but not the actual slow waves, were abolished if nifedipine was subsequently added to the superfusion fluid. Nifedipine was not added during recording of the jejunal sample. In one horse (00/345), the addition of nifedipine altered the appearance of the slow waves (Figure 42). As seen in Figure 42A, the slow waves in the absence of nifedipine had a biphasic appearance. Following the addition of nifedipine, a

typical slow wave with a fast upstroke and slower downstroke gradually became apparent (Figure 42B and C).

An attempt was always made to avoid adding nifedipine to the superfusion fluid prior to pelvic flexure recordings as the effect of this drug on electrical activity in the equine colon is unknown. However, if strong muscle contractions precluded stable cellular impalements, nifedipine was added.

Table 13: Resting membrane potential (RMP) and slow wave frequencies recorded in the longitudinal muscle layer of the small intestinal samples included in the study.

Horse	Number of cells impaled	RMP mV (mean)	Amplitude mV (mean)	Frequency per minute (mean)	Duration s (mean)
04/219	8	-35.0	5.5	9	6.6
00/345	6	-51.8	9.1	9	6.6
02/62	2	-19.0	4.0	8	7.5
04/391	5	-16.8	8.6	10	6.0
04/392	2	-12.4	4.0	12	5.0
04/569	3	-18.3	7.6	9	6.6

Key: RMP: Resting membrane potential

The recordings obtained from the pelvic flexure samples were markedly different from those made from the small intestine. Electrical activity was observed in all 7 horses included in the study although "typical" slow wave patterns as seen in the small intestine were not observed in the large intestine.

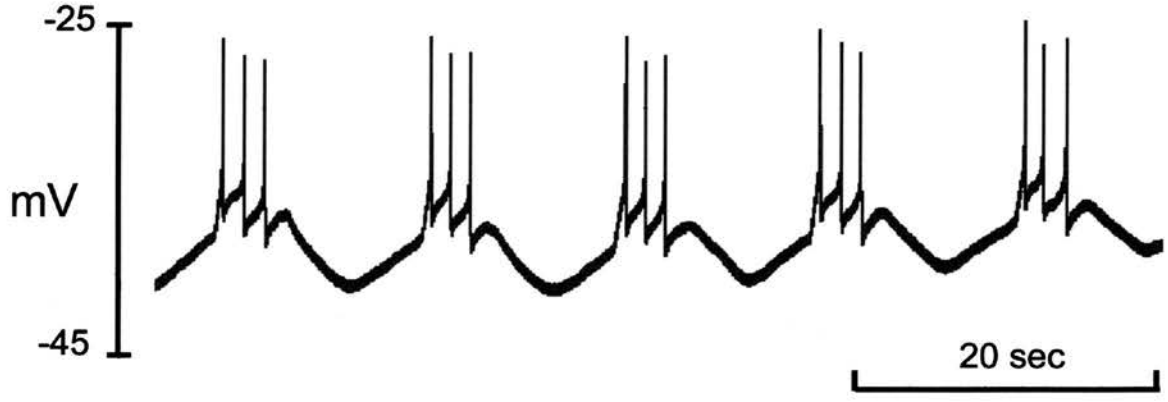


Figure 41: Intracellular recording from the longitudinal muscle layer (at a level of 10% through the longitudinal muscle layer from the myenteric border) of normal equine ileum. Associated spontaneous action potentials can be observed. No nifedipine was present.

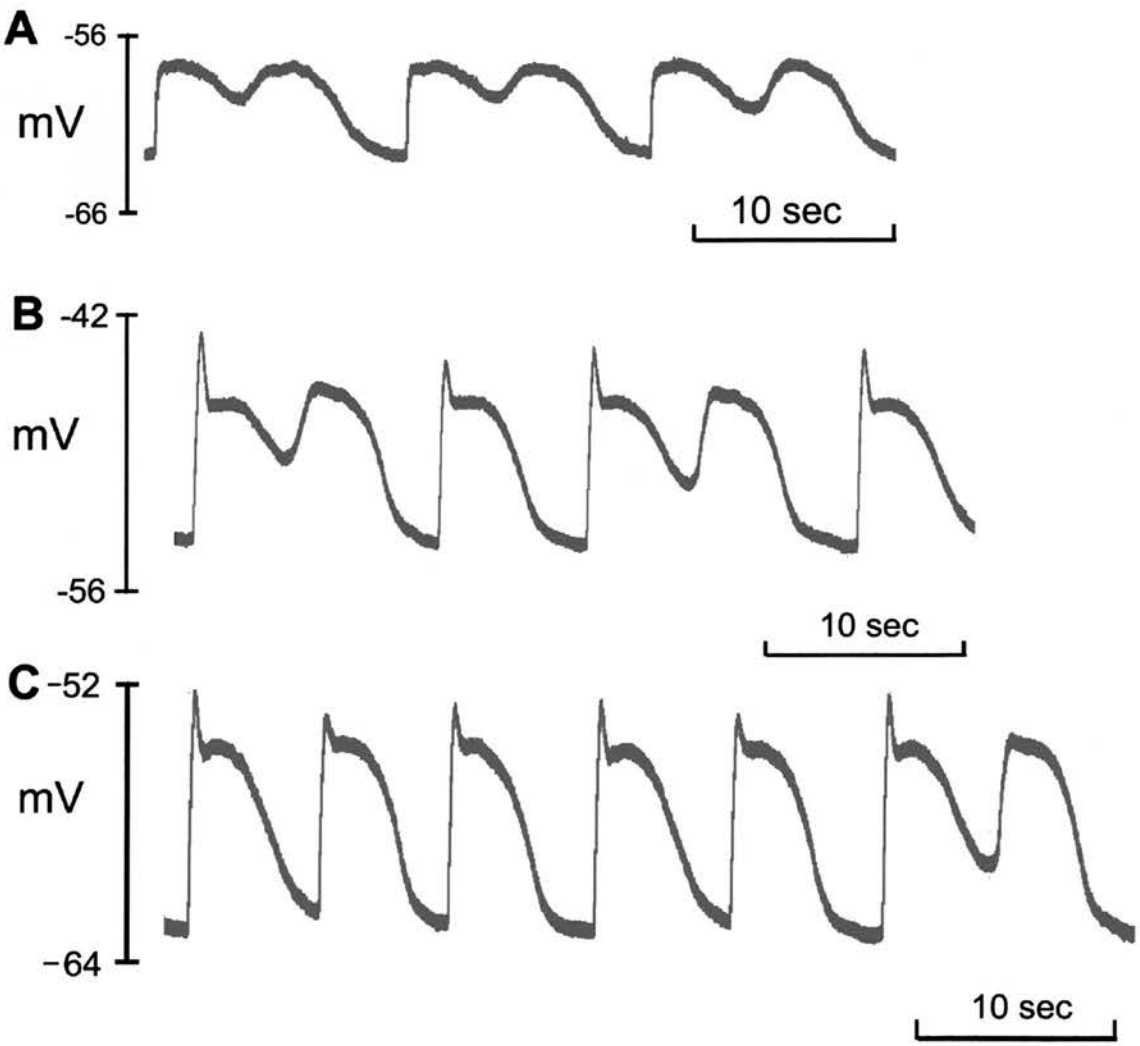


Figure 42: Intracellular recordings from the longitudinal muscle layer (at a level of 10% through the longitudinal muscle layer from the myenteric border) of equine ileum. A: Recording in the absence of nifedipine; B: Nifedipine has been present in the superfusion fluid for 2 minutes; C: Nifedipine has been present for 5 minutes.

In 6 of the 7 horses, two types of electrical events were recorded. The first of these was a long-duration depolarisation that occurred at a frequency of 2-12/minute. This event had a duration of 4-6 seconds and was characterised by a fast upstroke phase followed by a period where the voltage dropped gradually before a sudden, large drop in voltage occurred (Figure 43). In some of the traces, this appeared to be followed by a brief period of after-hyperpolarisation (Figure 43A), although due to a rather high background noise, this was difficult to measure accurately.

The second type of electrical event recorded was a transient, spike-like depolarisation with an amplitude ranging from 8-28 mV. This event showed considerable variation in frequency (11-110/minute) and its occurrence was regular, irregular or even in bursts. As illustrated in Figure 43, these transient depolarisations appeared to occur independently of the long-duration depolarisations described above as they appeared both between and superimposed on the latter. The recordings in one animal (00/345) demonstrated that the long-duration events could also occur in the absence of the fast, transient depolarisations (Figure 44).

All electrical events described were recorded in both the presence and absence of nifedipine in the superfusion fluid. Although the small sample size precluded statistical analysis, nifedipine did not appear to cause any significant change in the amplitude or frequency of the recorded waveforms.

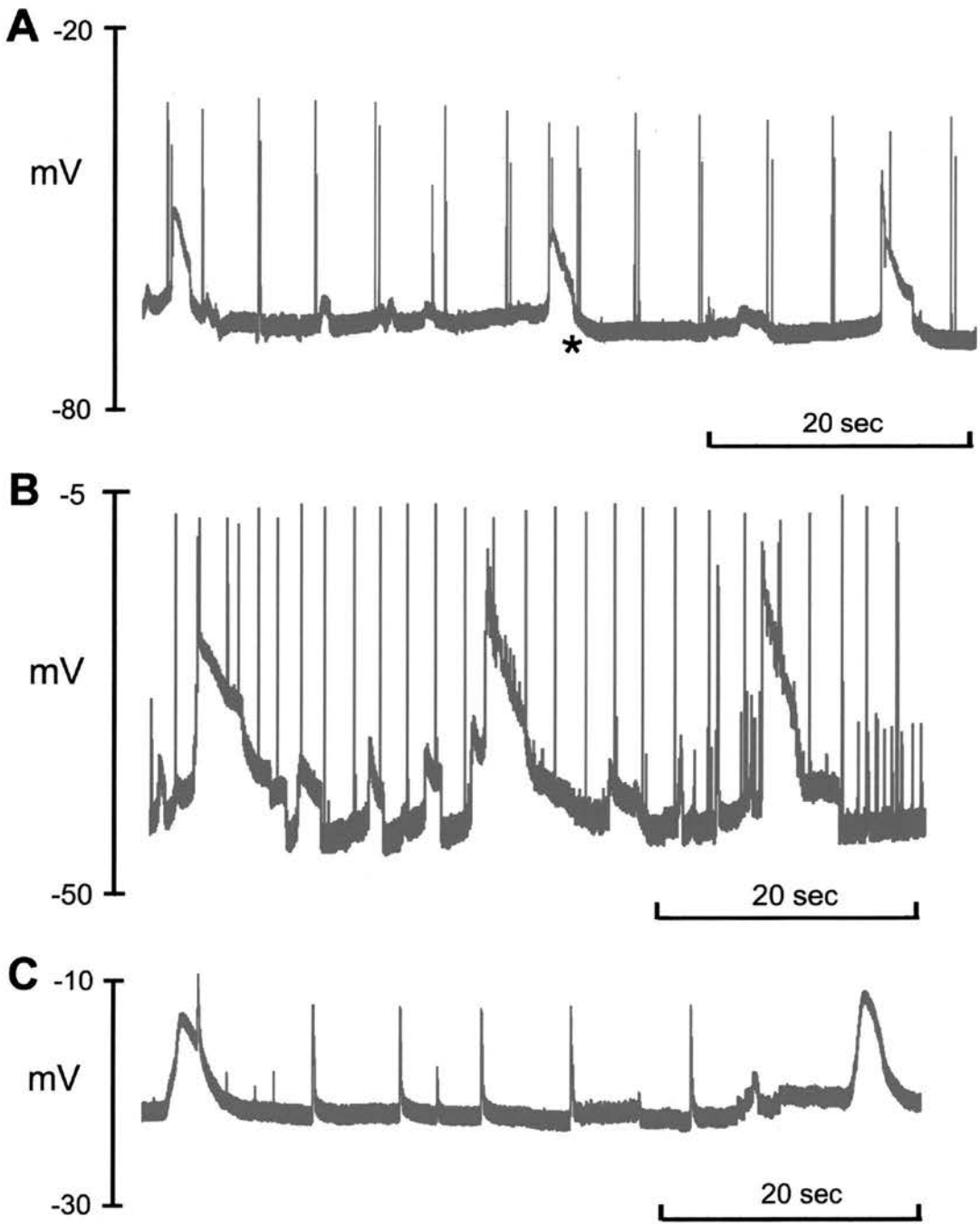


Figure 43: Intracellular recordings from the circular muscle layer (at a level <10% from the submucosal border) of pelvic flexure from three different horses. Long-duration depolarisations (3/minute) can be observed in all traces. Prominent spike-like activity can be observed between and superimposed on the long-duration depolarisations. Nifedipine was present in the superfusion fluid of sample C only. Asterisk (*) indicates a proposed point of hyperpolarisation.

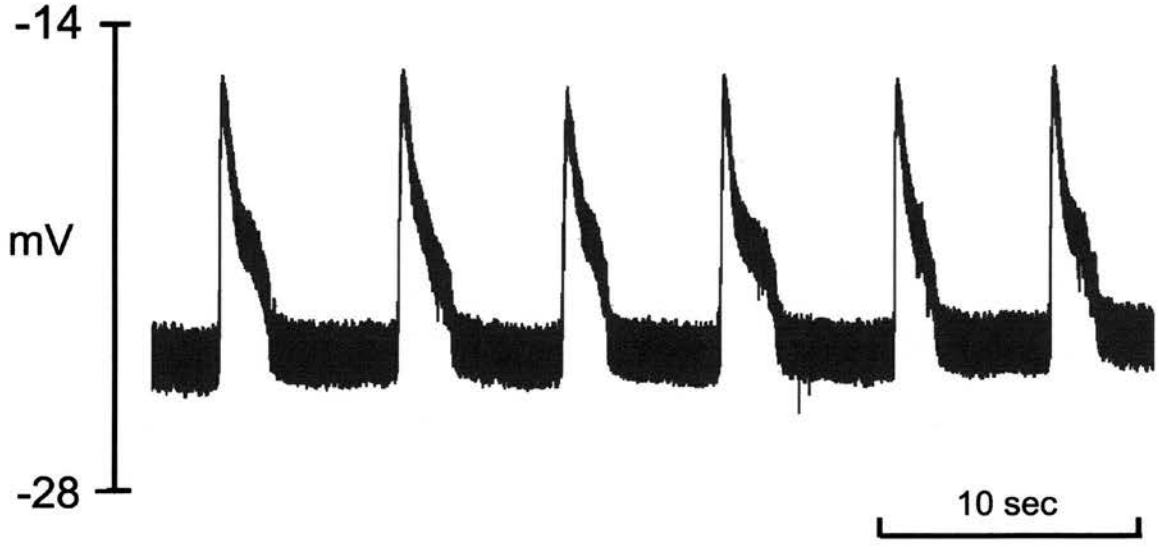


Figure 44: Intracellular recording from the circular muscle layer (at a level <10% from the submucosal border) of normal equine pelvic flexure. Long-duration depolarisations without the presence of spike-like activity can be observed. Nifedipine was present in the superfusion fluid.

Subjectively, there did not appear to be any difference in the quality of impalement between the different orientations of the circular muscle layer.

4.4 Discussion

The current investigation demonstrated slow wave activity in all of the small intestinal samples. These were included as positive controls and thereby validated the technique for the subsequent pelvic flexure recordings. The frequency of slow waves observed in the small intestinal samples corresponded well to that documented in the studies by Hudson *et al.* (2001c, 2002). Additionally, the RMP did not appear to have a significant effect on the amplitude of the slow waves which again is in agreement with the findings of Hudson *et al.* (2001c). It was noted that the recorded RMP was generally lower than that described by Hudson *et al.* (2001c, 2002). It is likely that this was a result of the variable quality of the impalements and glass electrodes. However as demonstrated, it was still possible to record electrical events including slow wave activity as described.

The addition of the L-type calcium channel blocker nifedipine did not appear to have an effect on the temporal characteristics of slow waves. Although caution is required due to the limited sample size in the current study, this differed from the findings by Hudson *et al.* (2001c) who demonstrated a reduction in frequency and increase in duration of slow waves following nifedipine administration. However, in one animal in the current study the appearance of slow waves did change markedly following the addition of nifedipine (Figure 42). Prior to this, a biphasic-like appearance of the slow waves was observed

which gradually returned to the more typical shape following the addition of nifedipine to the superfusion fluid. Bortoff (1965) suggested that two populations of pacemaker cells may be present in the feline jejunum following recording of waxing and waning slow wave patterns. Similar patterns were observed in the equine ileum (Hudson *et al.* 2001c). Perhaps the presence of two separate pacemakers may account for the recordings in the current study with one of these being sensitive to nifedipine allowing the second pacemaker to become dominant. It is also possible that the cellular impalement was not completely stable and that the addition of nifedipine facilitated the recording of the electrical activity. As described in the introduction of this thesis, the exact mechanism of slow wave generation by ICC has still not been fully determined. Earlier studies (Sanders 1996; Farrugia 1999) proposed that this event was initiated through spontaneous activation of low-threshold, T-type Ca^{2+} channels which caused depolarisation of the resting membrane potential activating L-type Ca^{2+} channels leading to amplification of the current. Recently, it has been proposed that the pacemaker current is initiated by voltage-independent, Ca^{2+}-inhibited, non-selective cationic conductance in the ICC (Koh *et al.* 1998; Thomsen *et al.* 1998; Koh *et al.* 2002; Koh *et al.* 2003; Sanders *et al.* 2004). Regardless of the initial mechanism, the opening of L-type Ca^{2+} channels is closely linked to smooth muscle cell contraction (Sanders 1996; Farrugia 1999). It is therefore possible that blocking of this channel and thereby action potential and associated muscle contraction would have stabilised the impalement to produce a more classical slow wave trace as seen in Figure 42C.

Slow wave activity was also recorded in the jejunum in the absence of nifedipine with a slightly higher frequency (12/minute) than that observed in the ileal samples (8-9/minute). Slow waves have not been previously recorded in the equine jejunum although Rakestraw *et al.* (2000) demonstrated small membrane potential oscillations that were abolished by nifedipine. It appears that the frequency of slow waves within a region is to some extent species- and location-specific (Furness and Costa 1987). It seems logical to have a higher slow wave frequency in the more proximal part of the small intestine to ensure the direction of flow remains in an oral-aboral direction. The frequencies recorded at different sites in the small intestine of different species including man, cat and dog have demonstrated that they do become gradually less frequent towards the caecum, although the lowest frequency recorded was in the stomach (Furness and Costa 1987). Further recordings from jejunal samples are needed in order to fully establish the slow wave frequency in this region as well as any effect nifedipine may have.

Several previous studies have investigated the motility patterns of the equine large colon, of which the majority have been extracellular myoelectrical studies (Sellers *et al.* 1979; Ruckebusch and Fioramonti 1980; Ruckebusch 1981; Roger and Ruckebusch 1987; Ruckebusch and Roger 1988; Merritt *et al.* 1995; Roussel *et al.* 2000). These have demonstrated a rather complex pattern that can be separated into different components. Although the nomenclature and definitions have differed, there appears to be agreement on some terms. This includes short and long spike bursts (SSB and LSB respectively) as well as migrating myoelectrical complexes (MMC). The former are specific patterns of

electrical response activity (or action potentials or spikes) that appear rhythmically in the stomach and small intestine (Szurszewski 1969; Sarna *et al.* 1982) as well as in the colon (Sarna *et al.* 1984; Sarna 1986). MMC represent waves of muscular and myoelectrical activity thought to mix interdigestive secretion with cellular and food debris and move them aborally (Szurszewski 1969). Sarna *et al.* (1984) concluded that the MMC of the dog colon were very different from those in the small intestine as the former migrate both orally and aborally, the effect of which is likely to maximise absorption of nutrients as well as finally resulting in the propulsion of feedstuff. All these complexes are part of a cyclical motor activity that ranges from 80-120 minutes in the small intestine (Szurszewski 1969; Code and Marlett 1975) whereas the duration in the colon is not clearly defined as it appears to display a more complex pattern involving retrograde contractile patterns (Sarna *et al.* 1984).

Varying durations of LSBs have been described in different studies although generally they seem to last more than 4 seconds in the horse (Ruckebusch and Fioramonti 1980; Merritt *et al.* 1995). Clusters of these have been demonstrated to form MMC in the pelvic flexure of the horse, although they may also present as a distinct, random activity (Merritt *et al.* 1995). The duration and activity of SSBs have also been described in the horse and generally they last less than 4-5 seconds (Ruckebusch and Fioramonti 1980; Merritt *et al.* 1995). It was proposed that these represent a basic pattern of myoelectrical spiking activity and that they may be associated with slow wave activity (Ruckebusch and Fioramonti 1980). Although it is clear that both LSB and SSB represent localised contractions that may result in a larger migrating contraction, the electrical counterpart

of these contractile complexes remains to be defined. Spencer *et al.* (2003) demonstrated that electrical slow waves were not required for the generation of MMC in the mouse small intestine. These complexes were still present in W/W^V mutant mice, which lack pacemaker ICC and slow waves, and it was proposed that the generation and propagation of MMC were the result of an intrinsic capability of the ENS and not related to slow waves (Spencer *et al.* 2003). A similar study has not been carried out in the colon.

Currently, slow wave activity has not been reported in the equine colon although several studies have described this activity in other mammalian species (Christensen *et al.* 1969; Durdle *et al.* 1983; Smith *et al.* 1987a, b; Rae *et al.* 1998; Plujà *et al.* 2001). These studies have demonstrated slow wave activity originating from the submucosal border of the circular muscle region (Sanders and Smith 1986b; Rae *et al.* 1998; Plujà *et al.* 2001). These studies also formed the basis for this particular investigation. It was hypothesised that if slow waves were present in the equine colon, it was possible that they too originate from this anatomical region.

In the present study, two main types of electrical events were apparent. The first of these was a slow depolarising electrical event that occurred at a frequency of 2-12/minute. This frequency was similar to the recorded colonic slow wave frequencies described in man, mice and rats (Durdle *et al.* 1983; Sanders *et al.* 1990; Smith *et al.* 1987a, b; Rae *et al.* 1998; Plujà *et al.* 2001; Ward *et al.* 2002). The shape of the recorded electrical events was similar to that of slow waves described in other species (Christensen *et al.* 1969;

Durdle *et al.* 1983; Sanders *et al.* 1990; Smith *et al.* 1987b; Rae *et al.* 1998; Plujà *et al.* 2001; Ward *et al.* 2002). However, it is acknowledged that further investigations are needed to establish the origin of the electrical activity observed. This also includes the investigation of the second, spike-like, electrical event recorded. Ward *et al.* (2002) demonstrated significant changes in the electrical activity in the colon between ganglionic and aganglionic mice. The aganglionic colon tissue samples were electrically quiescent compared to the spontaneous spiking activity of the controls. It is possible that the continuous and intermittent bursts of spike-like activity observed in the current study were excitatory junction potentials in the smooth muscle brought about by endogenously-released neurotransmitters. The addition of the neuronal blocker (Na^+ channel blocker) tetrodotoxin to the superfusion fluid may help answer this question.

All the recordings were made at no more than 10% from the submucosal border. As it was often difficult to obtain stable recordings of electrical activities, it was decided to limit these initial investigations to this anatomical area in order to obtain a level of standardisation. Unfortunately, the addition of nifedipine did not appear to markedly improve the stability of impalements. Subsequently, this often resulted in deep cellular impalements which likely accounted for a degree of background noise as seen in the illustrations. Similarly, because of these difficulties, the evaluation of other pharmacological agents such as a neuronal blocker tetrodotoxin (Na^+ channel blocker) to the superfusion fluid was not performed as it was considered likely that the interpretation of these results may have been unreliable. Future studies may benefit from the addition of the myosin light chain inhibitor wortmannin to the superfusion fluid

which may facilitate cellular impalement (Burke *et al.* 1996). There did not appear to be any difference in the orientation of the circular muscle layer to the quality of the impalement.

All animals included in this study were euthanised using barbiturates. It is not known what exact effect this has on slow wave activity although it seems unlikely that it influenced the electrical activities observed, as other studies have demonstrated apparently normal slow wave activity in tissue samples collected during surgery from animals that were anaesthetised using barbiturates (Sanders and Smith 1986a; Smith *et al.* 1987a, b; Sanders *et al.* 1990). Furthermore, all tissue samples underwent an equilibration period of at least 1 hour during which they were continuously superfused with fresh, oxygenated Krebs solution and it seems reasonable to assume that any traces of barbiturates would have been flushed away during this process.

The difficulty in obtaining fresh tissue samples meant that horses both with and without intestinal disease had to be included in the study. However, it was interesting to note that there did not appear to be any marked difference in the patterns observed in these two groups of horses. It is clear from this pilot study that further studies are needed to answer some of these many questions that have arisen. It also emphasises the complexity of motility patterns in the equine large colon.

5 DISCUSSION AND CONCLUSIONS

5.1 General discussion

5.1.1 Immunohistochemical studies

This investigation explored ICC distribution and density in the intestinal tract of the developing equine foetus as well as in horses and donkeys with intestinal disease. Neuronal densities in the intestinal tract of healthy and diseased horses and donkeys were also evaluated.

It was important to obtain information on the ontogeny of ICC as a foundation now exists for the interpretation of samples collected from diseased equine neonates. Abnormal ICC development in human neonates has been implicated in conditions such as meconium impaction (Yoo *et al.* 2002), hypertrophic pyloric stenosis (Langer *et al.* 1995), anorectal malformations (Kenny *et al.* 1998a) and transient neonatal intestinal pseudo-obstruction (Kenny *et al.* 1998b; Yamataka *et al.* 1998). It seems reasonable to assume that abnormal ICC development may also be involved in some of the motility disorders seen in the foal, particularly in the immature and dysmature animal. There are not many conditions where a clear link, between pathology and its effect on motility, has been demonstrated. One such condition is "lethal white foal syndrome," the equine equivalent of Hirschprung's disease. Two separate studies have demonstrated how a mutation in the *endothelin-B* receptor gene in white progeny of overo spotted horses

results in ileocolonic aganglionosis and the effect this has on motility in the affected intestinal segment (Metallinos *et al.* 1998; Santschi *et al.* 1998). It is hoped that once the role of ICC in generating and maintaining intestinal motility patterns in the equine neonate has been established, the precise effect of disruption or immaturity of ICC can be determined as this may eventually provide a precise therapeutic target.

Functional studies in the adult horse are also warranted in order to further interpret the significance of the findings in the current investigation demonstrating a reduction in ICC densities in horses with an obstructive disorder of the large intestine. Studies in other mammalian species have demonstrated that disruptions to ICC networks will affect slow wave patterns (Ward *et al.* 1994; Torihashi *et al.* 1995, 1997; Ward *et al.* 1997; Kenny *et al.* 1998b; Mikkelsen *et al.* 1998) and it seems reasonable to assume that similar disruptions may also occur in the equine large colon. Hudson *et al.* (2002) demonstrated a significant reduction in slow wave frequencies in the ileum of horses with EGS compared to control animals using intracellular recording techniques. These horses also had reductions in their ICC densities as demonstrated by c-Kit immunohistochemistry (Hudson *et al.* 2002). The precise effect reduced c-Kit immunoreactivity has on slow wave activity in the equine colon still needs to be established. However, before interpretation of diseased tissue is possible, normal slow wave patterns in the large intestine need to be determined. Currently, the majority of motility investigations of this region in the horse have been extracellular myoelectrical studies (Sellers *et al.* 1979; Ruckebusch and Fioramonti 1980; Ruckebusch 1981; Roger and Ruckebusch 1987;

Ruckebusch and Roger 1988; Merritt *et al.* 1995; Roussel *et al.* 2000) but the correlation of these activities to slow wave patterns still needs to be established.

The current immunohistochemical study also raised a question of cause and effect as a number of horses with a reduction in ICC densities had a history of previous colic episodes. It is possible that some of these horses already had disruptions to their ICC networks creating abnormal motility patterns and thereby making these animals more prone to developing colic, although when or how these changes occurred is impossible to determine. From a limited number of studies performed, it has been demonstrated that ICC are susceptible to both hypoxic and inflammatory changes in their environment (Christensen and Rick 1994; Lu *et al.* 1997; Der *et al.* 2000; Wang *et al.* 2002). It is possible that intestinal inflammatory conditions, such as those related to worm infestation, could have been present at an earlier point resulting in ICC pathology and disruption to their networks. As it was not always possible to obtain a comprehensive previous medical history this has to remain a speculation although nevertheless a possibility. Importantly, it appears that ICC have some capacity for repair following injury (Der *et al.* 2000; Chang *et al.* 2001; Wang *et al.* 2002). Der *et al.* (2000) reported that following experimental infection of mice small intestine with *Trichinella spiralis* infection, abnormal motility patterns were eventually restored but it took up to 60 days to do so. Whether ICC function can be fully restored following a severe insult, remains to be fully established.

It is possible that some of the horses in the current study experienced both hypoxia and inflammation of the affected segment of intestine during the current colic episode thereby raising the possibility that ICC injury could have occurred at this point rather than by a previous insult. This raised the question of how long it may take for ICC damage to occur. It was proposed that the time interval from the first sign of colic observed by the owner to surgery and sample collection was too short in the majority of cases to account for the changes observed and hence they may already have been present prior to sample collection. However, it was acknowledged that this time interval may have been longer as subtle clinical signs may not have been immediately observed by the owner. Additionally, there may have been ongoing intestinal pathology some time prior to onset of clinical signs. Although this study has raised several interesting questions, it is hoped that it has also added to the literature on possible causes of intestinal motility dysfunction in the horse. It is clear that further investigations are needed in order to answer some these.

The importance of having an intact ICC network was further demonstrated in the study on two recovered chronic EGS that had apparently normal intestinal motility. These horses had apparently normal ileal ICC densities in the presence of a severely reduced enteric neuronal network. As already discussed, ICC appear to have some capacity for repair following injury and therefore it was proposed that either the ICC in these horses were injured and subsequently recovered, and/or they were not severely disrupted during the initial insult to account for these findings. Hudson *et al.* (2001c) demonstrated in an immunohistochemical study that ICC densities were reduced in horses with chronic

EGS, therefore it is possible that some degree of injury and subsequent repair happened in these two horses. As laparotomy is generally contraindicated in chronic cases where nursing is contemplated (Milne *et al.* 1994), it is very difficult to obtain samples collected during the initial disease process and indeed following recovery when the animal is euthanised for reasons not relating to the GI tract. Although it is appreciated that it may take time for such samples to be accumulated, it is nevertheless an important task to help shed light on these intriguing findings.

The ICC densities and distribution in donkeys demonstrated many similarities to that observed in the normal adult horse. However, reduced densities were not observed in diseased animals compared to the controls. It is possible that if more animals were examined, a significant difference would become apparent. It is equally possible that the problems these animals experienced were not caused by an intrinsic motility disorder and therefore other factors such as management practices must be considered. This appears particularly important in view of the typical case history of these animals where impaction colics generally occur during winter housing which involves less exercise and a higher proportion of fibrous feed (such as straw) for these donkeys. In addition, this population of donkeys were all aged and so the dental health of the animals was also considered. This failed to demonstrate a significant association between poor dentition and the incidence of colic although it is still proposed that the problem this particular herd experienced was more likely related to management practices rather than intrinsic motility dysfunction of the animals. These conclusions are in agreement with a previous survey investigating this problem in the same donkey population (Duffield *et al.* 2002).

Identification of ICC has been made relatively easy in recent years through immunohistochemical labelling of the c-Kit receptor (Ward *et al.* 1994; Huizinga *et al.* 1995; Torihashi *et al.* 1995). Currently, targeting this receptor tyrosine kinase is the only way of identifying ICC in the adult using immunohistochemistry. This may present a problem when a tissue sample displays reduced c-Kit immunoreactivity as it may not necessarily mean that the whole cell has been disrupted. Furthermore, as demonstrated in the developmental study, the most luminal part of the distal small colon appeared not completely colonised by ICC at the time of birth based on c-Kit immunoreactivity. It is possible of course the ICC are present but that the c-Kit receptor is too immature for an antibody to recognise it. For these reasons, the usefulness of an alternative marker, vimentin, was investigated. Vimentin is an intermediate filament which represents a diverse group of fibrous proteins expressed in the cytoplasm of higher eukaryotic cells, including ICC (Fuchs and Cleveland 1998). Unfortunately, vimentin is not exclusive to ICC and so both enteric neurons as well as smooth muscle cells were additionally labelled hence it was difficult to make out individual ICC in the tissue samples examined. This was particularly true for the large colon samples. However, where ICC density was greatest, along the myenteric plexus region of the small intestine, the immunoreactivity of vimentin mirrored that of c-Kit indicating that it may be of use in some cases. Further studies investigating tissue samples collected from diseased horses with reduced c-Kit immunoreactivity in this region are needed to fully evaluate this possibility. Additionally, alternative markers may be explored in order to identify immature ICC in the distal small colon of equine neonates. This is particularly important as the morphological features of ICC are similar to several cell types within the

muscularis externa including both smooth muscle cells and fibroblasts in the developing intestinal tract making them difficult to identify using ultrastructural analysis (Thuneberg 1982; Faussone-Pellegrini 1984, 1987; Faussone-Pellegrini *et al.* 1985, 1996).

As described in the introduction of this thesis, the ENS also represents an essential component of normal intestinal motility function. Two previous studies, based on H&E evaluation, have demonstrated a significant reduction in myenteric neuronal densities in horses with acute and chronic obstructive disorders of the caecum (Schusser *et al.* 2000) and large colon (Schusser and White 1999). It was proposed that these changes may have been a reason for the increased risk of recurrent colic from colonic or caecal dysfunction in the horses examined (Schusser *et al.* 2000). The current investigation failed to demonstrate a significant reduction in neuronal densities in horses and donkeys with intestinal disease using the neuronal immunohistochemical marker protein gene product 9.5 (PGP 9.5). However, two horses in the colic study, of which one had a history of repeated colic episodes, did demonstrate reduced neuronal densities and it is possible that this was part of an intrinsic motility problem experienced by these particular animals.

5.1.2 Molecular studies

This study demonstrated that transcription levels of the c-*kit* gene encoding the c-Kit receptor did not change in horses with intestinal disease compared to a control group.

However, as demonstrated in the previous immunohistochemical study, a significant reduction in the density of c-Kit-immunoreactive ICC was observed in horses with an obstructive lesion of the large intestine. This discrepancy opened up a number of questions relating to the regulation of c-Kit protein production and expression.

Given these results, it was proposed that regulation of protein production was likely to occur at a point downstream to transcription, possibly at a translational level. This was an unexpected finding as gene regulation occurs primarily at a transcriptional level (Beyersmann 2000). Naturally, the methodology had to be scrutinised and although some criticisms could be made, the results were considered reliable. This was largely due to the high level of accuracy and reproducibility that real-time RT-PCR offers (Heid *et al.* 1996).

The control of protein expression is an extremely complex process of which there is a significant amount of ongoing research trying to unravel some of these regulatory processes (Boerner *et al.* 2003; Ossovskaya and Bunnett 2004). Many of these are associated with responses to changes in the cellular environment such as inflammation (Ossovskaya and Bunnett 2004). As yet, there is no specific information on regulatory responses of c-Kit although it may be possible to draw parallels from work that is ongoing on other members of the oncogene family (Boerner *et al.* 2003). Because of the complexity of this work, it may seem outside the scope of a clinical investigation such as this to investigate these regulatory responses in detail. However, there are avenues of investigations that are possible to pursue in order to obtain important information with

the clinical material available. Firstly, the results of this study describing a reduction in c-Kit immunoreactivity in horses with a large colon obstructive disorder supported the findings of the earlier immunohistochemical investigation. This indicates that it would be sensible to focus future studies on the c-Kit protein and the changes that occur to it under conditions of cellular stress. It would be useful to obtain an accurate estimate of c-Kit protein present through Western blot analysis from collected tissue samples from both normal and diseased animals. This is an electroblotting method in which proteins are transferred from a gel to a thin, rigid support (nitrocellulose) and detected by binding of labelled antibody, in this case c-Kit. A parallel ultrastructural and immunohistochemical assessment of the tissue samples would assess how protein quantification compares with the extent of ICC disruption and c-Kit immunoreactivity.

Recently, Rowe *et al.* (2003) demonstrated a high number of apoptotic cells in the intestine from horses with obstructing or strangulating lesions in the small intestine and large colon using a terminal deoxynucleotide transferase mediated dUTP nick end labelling (TUNEL) technique. The decision of a cell to undergo apoptosis, i.e. physiologic or programmed cell death, can be influenced by a wide variety of regulatory stimuli (Thompson 1995; Rudin and Thompson 1997; Dragovich *et al.* 1998). In the past decade, it has become clear that the regulatory mechanisms controlling programmed cell death are as fundamental, and as complex, as those regulating cell proliferation (Rudin and Thompson 1997). It is possible that ICC also undergo some degree of apoptosis during periods of cellular stress. One of the morphologic hallmarks of apoptosis includes membrane blebbing (Kerr *et al.* 1972). This ultrastructural feature has been described in

ICC in the dog and mouse intestine following experimentally induced inflammation (Lu *et al.* 1997; Der *et al.* 2000; Wang *et al.* 2002). If apoptosis also affects ICC during inflammatory conditions, it may offer an additional explanation on why a significant proportion of horses with reduced ICC densities experienced repeated colic episodes. It was proposed earlier that ICC may be severely and permanently injured during cellular insults. It is also possible that inflammatory mediators could induce ICC apoptosis. This is an area that warrants further investigation.

5.1.3 Electrophysiological studies

The previous sections have described immunohistochemical changes demonstrating a reduction in c-Kit-immunoreactive ICC in the pelvic flexure of horses with an obstructive disorder of the large intestine. Because of the functional importance of the c-Kit receptor, it was proposed that the reduced immunoreactivity observed would be associated with abnormal motility patterns. Slow wave activity has not been previously described in the large intestine of the horse although Hudson *et al.* (2001c, 2002) described these patterns in the equine ileum. Currently, the majority of motility investigations in the equine large colon have been extracellular myoelectrical studies (Sellers *et al.* 1979; Ruckebusch and Fioramonti 1980; Ruckebusch 1981; Roger and Ruckebusch 1987; Ruckebusch and Roger 1988; Merritt *et al.* 1995; Roussel *et al.* 2000) but the correlation of these activities to slow wave patterns still needs to be established. The current investigation focused on the pelvic flexure area as this anatomical area is thought to be an important motility control centre in the horse and is also important

clinically as many equine motility disorders, such as impactions, involve this region (Lowe *et al.* 1980; Sellers *et al.* 1982, 1984; White 1990; Lopes and Pfeiffer 2000).

The current investigation obtained traces of electrical activity in all animals examined. Slow wave activity was observed in all small intestinal samples, including one jejunal sample that was examined. The frequencies of the slow waves observed in the ileal samples were similar to those recorded by Hudson *et al.* (2001c). Rakestraw *et al.* (2000) demonstrated small membrane potential oscillations that were abolished by nifedipine in the equine jejunum. Although slow wave activity was recorded in the jejunum of one horse in the current study, the effect of nifedipine was not evaluated in this case.

Although it could not be concluded that the electrical pattern observed in the pelvic flexure samples were indeed slow waves, they were highly suggestive of these especially when compared to those seen in other mammalian species (Christensen *et al.* 1969; Durdle *et al.* 1983; Sanders and Smith 1986a, b; Smith and Sanders 1987a, b; Rae *et al.* 1998; Pluja *et al.* 2001). However, to further investigate the electrical activity in this region it is necessary to obtain better quality tissue impalements. The addition of the myosin light chain kinase inhibitor Wortmannin (Burdyga and Wray 1998) may help improve this. In order to evaluate any neuronal influence on the electrical recordings, it is important that future investigations include the addition of inhibitors such as the sodium-channel blocker tetrodotoxin.

Once the technique has been fully established, it should be possible to obtain stable and repeatable electrical recordings from the equine large colon. This may facilitate the investigation of samples from diseased animals as well as samples collected from the distal small colon of the full term foal. The latter may help establish if the slow wave activity in this region is indeed absent or rudimentary and how this may change in the older foal. Furthermore, it would be interesting to investigate the activities of the taenial bands to obtain further information on their importance in intestinal motility.

5.2 Conclusions

- The development and distribution of ICC in the equine foetus was described. This will form a basis for future immunohistochemical investigations of neonates with intestinal motility disorders.

- A significant reduction in ICC density was demonstrated in horses with an obstructive disorder of the large colon and it was proposed that this was linked to the motility problems of these animals. A similar reduction could not be established in a population of donkeys although it was proposed that management factors rather than intrinsic motility dysfunction was the main cause of the intestinal disease experienced by these animals.

- Examination of two recovered chronic EGS cases demonstrated apparently normal ICC densities in small intestinal tissue samples. It was proposed that this

could offer an explanation for the normal intestinal function in these horses in the absence of an intact enteric neuronal network.

- The effectiveness of an alternative marker of ICC, vimentin, was suggested to have some possible use in selected small intestinal samples although limited use in large intestinal samples.

- Immunohistochemical investigation of neuronal densities using a PGP 9.5 marker failed to demonstrate any significant changes in horses and donkeys with intestinal disease compared to the control groups.

- A comparative analysis of the c-*kit* gene transcription levels encoding the c-Kit receptor failed to demonstrate a significant difference in normal versus diseased animals although a parallel immunohistochemical study confirmed the findings of the earlier, separate immunohistochemical study. It was proposed that the control of c-Kit protein production lies primarily at a point downstream to the transcriptional level.

- Intracellular recordings of pelvic flexure tissue samples demonstrated electrical activities some of which may be consistent with slow wave activity.

5.3 Future studies

A basis has been formed for future immunohistochemical studies investigating possible abnormalities in ICC development and distribution in equine neonates with intestinal motility disorders. A good collaborative link has been established with Rossdale and Partners in Newmarket who have the case load and interest to help further this investigation. Furthermore, as part of investigating motility disorders in this age group, the development of the ENS should be described in order to evaluate if abnormalities of this system could also be involved in some of the many disorders seen in the foal. It would be of benefit to obtain samples from even younger foetuses as it is likely that the colonisation of neurons commence prior to that of ICC as discussed earlier. This means that ideally samples should be collected from animals towards the end of their first trimester in order to include the initial neuronal colonisation of the intestinal tract. This would also be of use to confirm that ICC colonisation follow that of neurons.

Two separate studies demonstrated a reduction in c-Kit-immunoreactive ICC in pelvic flexure samples collected from adult horses with an obstructive lesion of the large intestine. The follow-up questions from these include which processes are involved, what is the exact damage to ICC and what effect does this have on ICC function? It may be necessary to create an *in vitro* environment that can mimic a hypoxic or inflammatory insult to a healthy intestinal tissue sample. Once the tissue sample has been subjected to these insults, intracellular electrophysiological recordings as well as ultrastructural and parallel immunohistochemical analysis could be performed.

As an excellent collaborative link has also been established with The University of Liverpool, ultrastructural and parallel immunohistochemical evaluation of tissue samples collected from diseased animals could be performed. In addition, Western blot analysis of these samples would provide a comparative and objective measurement of c-Kit. It would also be of great interest to evaluate if a double-labelling method of ICC using the TUNEL technique and c-Kit could be established. This could help determine if the intestinal disease process involves a significant induction of apoptosis which may help answer the cause and effect question discussed in the earlier sections.

It is hoped that the work included in this thesis has helped expand our knowledge on the involvement of ICC in maintaining normal intestinal motility patterns as well as the role they may play in equine motility disorders.

6 BIBLIOGRAPHY

Argenzio RE, Lowe JE, Pickard DW, Stevens CE (1974) Digesta passage and water exchange in the equine large intestine. *J. Phys.* **226**, 1035-1042.

Argenzio RE (1975) Functions of the equine large intestine and their interrelationship in disease. *Cornell Vet.* **65**, 303-330.

Balemba OB, Mbassa GK, Semuguruka WD, Assey RJ, Kahawa CKB, Hay-Schmidt A, Dantzer V (1999) The topography, architecture and structure of the enteric nervous system in the jejunum and ileum of cattle. *J. Anat.* **195**, 1-9.

Belloli C, Arioli F, Beretta C, Madonna M (1994) *In vitro* effects of tachykinins on the smooth musculature of horse gut. *J. vet. Pharmacol. Therap.* **17**, 379-383.

Berne RM, Levy MN (1993) Chapter 8: Gastrointestinal Motility. In: *Physiology*. Eds: Berne, R. M. and Levy, M. N. Philadelphia, PA: Mosby International. pp615-651.

Beyersmann D (2000) Regulation of mammalian gene expression. *EXS* **89**, 11-28.

Blikslager AT, Bowman KF, Have ML, Tate Jr LP, Bristol DG (1992) Pedunculated lipomas as a cause of intestinal obstruction in horses: 17 cases (1983-1990). *J. Am. vet. med. Ass.* **201**, 1249-1252.

Boeke J (1949) The sympathetic endforms, its synaptology, the interstitial cells, the periterminal network, and its bearing on the neurone theory. Discussion and critique. *Acta Anatom.* **8**, 18-61.

Boerner JL, Danielsen A, Maihle NJ (2003) Ligand-independent oncogenic signaling by the epidermal growth factor receptor: v-ErbB as a paradigm. *Exp. Cell Res.* **284**, 111-121.

Bolton TB, Prestwich SA, Zholos AV, Gordienko DV (1999) Excitation-contraction coupling in gastrointestinal and other smooth muscles. *Ann. Rev. Physiol.* **61**, 85-115.

Bornstein JC, Furness JB, Kunze WAA (1994) Electrophysiological characterization of myenteric neurons: how do classification schemes relate? *J. Autonom. nerv. Syst.* **48**, 1-15.

Brookes SJH, Ewart WR, Wingate DL (1987) Intracellular recordings from the myenteric neurones in the human colon. *J. Phys.* **390**, 305-318.

Burdyga TV, Wray S (1998) The effect of inhibition of myosin light chain kinase by Wortmannin on intracellular [Ca2+], electrical activity and force in phasic smooth muscle. *Pflugers Arch.* **436**, 801-803.

Burke EP, Gerthoffer WT, Sanders KM, Publicover NG (1996) Wortmannin inhibits contraction without altering electrical activity in canine gastric smooth muscle. *Am. J. Phys.* **270**, C1405-C1412.

Burns GA, Karcher LF, Cummings JF (1989) Equine myenteric ganglionitis: A case of chronic intestinal pseudo-obstruction. *Cornell Vet.* **80**, 53-63.

Burns GA, Cummings JF (1991) Equine myenteric plexus with special reference to the pelvic flexure pacemaker. *Anatom. Rec.* **230**, 417-424.

Burns GA (1992) The teniae of the equine intestinal tract. *Cornell Vet.* **82**, 187-212.

Burns GA, Cummings JF (1993) Neuropeptide distributions in the colon, cecum, and jejunum of the horse. *Anatom. Rec.* **236**, 341-350.

Cajal SR (1883) Sur les ganglions et plexus nerveux de l'intestin. *CR Soc. Biol. (Paris)* **45**, 217-223.

Cajal SR (1911) Histologie du système nerveux de l'homme et des vértebrés. Volume 2. Paris: Maloine, 891-942.

Chang I-Y, Glasgow NJ, Takayama I, Horiguchi K, Sanders KM, Ward SM (2001) Loss of interstitial cells of Cajal and development of electrical dysfunction in murine small bowel obstruction. *J. Phys.* **536**, 555-568.

Christensen J, Caprilli R, Lund GF (1969) Electrical slow waves in circular muscle of cat colon. *Am. J. Phys.* **217**, 771-776.

Christensen J, Rick GA (1994) Interstitial cells of Cajal in the rat colon are damaged by mild hypoxia. *J. Auton. Nerv. Syst.* **48**, 175-180.

Code CF, Marlett JA (1975) The interdigestive myo-electric complex of the stomach and small bowel of dogs. *J. Physiol.* **246**, 289-309.

Cohen ND, Matejka PL, Honnas CM, Hooper RN (1995) Case-control study of the association between various management factors and development of colic in horses. *J. Am. vet. med. Ass.* **206**, 667-673.

Cohen ND, Peloso JG (1996) Risk factors for history of previous colic and for chronic, intermittent colic in a population of horses. *J. Am. vet. med. Ass.* **208**, 697-703.

Cohen ND, Gibbs PG, Woods AM (1999) Dietary and other management factors associated with colic in horse. *J. Am. vet. med. Ass.* **215**, 53-60.

Cook RD, Burnstock G (1976) The ultrastructure of Auerbach's plexus in the guinea-pig. II. Non-neuronal elements. *J. Neurocyt.* **5**, 195-206.

Cooke HJ (1994) Submucosal neural circuitry controlling ion transport and its relation to motility. In *Advances in the Innervation of the Gastrointestinal Tract*, Eds. Holle, G and Wood JD; Elsevier, Amsterdam, pp433-441.

Cortesini C, Cianchi F, Infantino A, Lise M (1995) Nitric oxide synthase and VIP distribution in enteric nervous system in idiopathic chronic constipation. *Dig. Dis. Sci.* **40**, 2450-2455.

Cottrell DF, McGorum BC, Pearson GT (1999) The neurology and enterology and of equine grass sickness: a review of basic mechanisms. *Neurogastroenterol. Motil.* **11**, 79-92.

Daniel EE, Posey-Daniel V (1984) Neuromuscular structures in opossum esophagus: Role of interstitial cells of Cajal. *Am. J. Phys.* **246**, G305-G315.

Daniel EE, Wang Y-F, Cayabyab FS (1998) Role of gap junctions in structural arrangements of interstitial cells of Cajal. *Am. J. Phys.* **274**, G1125-G1141.

Der T, Bercik P, Donnelly G, Jackson T, Berezin I, Collins SM, Huizinga JD (2000) Interstitial cells of Cajal and inflammation-induced motor dysfunction in the mouse small intestine. *Gastroenterology* **119**, 1590-1599.

Dever TE (2002) Gene-specific regulation by general translation factors. *Cell* **108**, 545-556.

Doxey DL, Gilmour JS, Milne EM (1991) A comparative study of normal equine populations and those with grass sickness (dysautonomia) in eastern Scotland. *Equine vet. J.* **23**, 365-369.

Doxey DL, Pogson DM, Milne EM (1992) Clinical equine dysautonomia and autonomic neuron damage. *Res. Vet. Sci.* **53**, 106-109.

Doxey DL, Pearson GT, Milne EM, Gilmour JS, Chisholm HK (1995) The equine enteric nervous system- neuron characterization and distribution in adult and juveniles. *Vet. Res. Comm.* **19**, 433-449.

Doxey DL, Johnston P, Hahn C, Reynolds J (2000) Histology in recovered cases of grass sickness. *Vet. Rec.* **146**, 645-646.

Dragovich T, Rudin CM, Thompson CB (1998) Signal transduction pathways that regulate cell survival and cell death. *Oncogene* **17**, 3207-3213.

Drew JS, Murphy RA (1997) Actin isoform expression, cellular heterogeneity, and contractile function in smooth muscle. *Can. J. Physiol. Pharmacol.* **75**, 869-877.

Ducharme NG, Hackett RP, Dcharme GR, Long S (1983) Surgical treatment of colic. Results in 181 horses. *Vet. Surg.* **12**, 206-209.

Duffield HF, Bell N, Henson FMD (2002) Factors associated with impactive colic in the donkey. Abstract, 7th International Equine Colic Research Symposium, Manchester, UK, pp122-123.

Duncan JL, Pirie HM (1972) The life cycle of *Strongylus vulgaris* in the horse. *Res. vet. Sci.* **13**, 374-379.

Durdle NG, Kingma YJ, Bowes KL, Chambers MM (1983) Origin of slow waves in the canine colon. *Gastroenterology* **84**, 375-382.

Duthie HL, Kirk D (1978) Electrical activity of human colonic smooth muscle *in vitro*. *J. Phys.* **283**, 319-330.

Dyce KM, Hartman W (1973) An endoscopic study of the caecal base of the horse. *Tijdschr. Diergeneesk*, **98**, 957-963.

Dyce KM, Sack WO, Wensing CJ (1996) The abdomen of the horse. In: *Textbook of veterinary anatomy.* Eds: Dyce KM, Sack WO, Wensing CJ. Philadelphia, WB Saunders, pp 503-521.

Edwards GB, Proudman CJ (1994) An analysis of 75 cases of intestinal obstruction caused by pedunculated lipomas. *Equine vet. J.* **26**, 18-21.

El-Sharkawy TY (1983) Electrical activities of the muscle layers of the canine colon. *J. Phys.* **342**, 67-83.

Epperson A, Hatton WJ, Callaghan B, Doherty P, Walker RL, Sanders KM, Ward SM, Horowitz B (2000) Molecular markers expressed in cultured and freshly isolated interstitial cells of Cajal. *Am. J. Phys.* **279**, C529-C539.

Farrugia G (1999) Ionic conductances in gastrointestinal smooth muscles and interstitial cells of Cajal. *Annu. Rev. Physiol.* **61**, 45-84.

Faussone-Pellegrini MS (1984) Morphogenesis of the special circular muscle layer and of the interstitial cells of Cajal related to the plexus muscularis profondus of mouse intestinal muscle coat: An EM study. *Anat. Embryol.* **169**, 151-158.

Faussone-Pellegrini MS, Matini P, Stach W (1985) Differentiation of enteric plexuses and interstitial cells of Cajal in the rat gut during pre- and postnatal life. *Acta anat.* **155**, 113-125.

Faussone-Pellegrini MS (1987) Cytodifferentiation of the interstitial cells of Cajal of mouse colonic circular muscle layer. *Acta anat.* **128**, 98-109.

Faussone-Pellegrini MS, Matini P, Stach W (1996) Differentiation of enteric plexuses and interstitial cells of Cajal in the rat gut during pre- and postnatal life. *Acta anat.* **155**, 113-125.

Faussone-Pellegrini MS, Fociani P, Buffa R, Basilisco G (1999) Loss of interstitial cells and a fibromuscular layer on the luminal side of the colonic circular muscle presenting as megacolon in the adult patient. *Gut* **45**, 775-779.

Flanagan JG, Chan DC, Leder P (1991) Transmembane form of the *kit* ligand growth factor is determined by alternative splicing and is missing in the Sl^{d} mutant. *Cell* **64**, 1025-1035.

Foxx-Orenstein AE, Kuemmerle JF, Grider JR (1996) Distinct 5-HT receptors mediate the peristaltic reflex induced by mucosal stimuli in human and guinea pig intestine. *Gastroenterology* **111**, 1281-1290.

Fuchs E, Cleveland DW (1998) A structural scaffolding of intermediate filaments in health and disease. *Science* **279**, 514-519.

Furness JB, Costa M (1980) Types of nerves in the enteric nervous system. *Neuroscience* **5**, 1-20.

Furness JB, Costa M (1987) Arrangement of the enteric plexuses. In: *The enteric nervous system.* Churchill Livingstone Inc. 1560 Broadway, New York, NY, 10036, pp6-26.

Galli SJ, Tsai M, Wershil, BK (1993) The *c-kit* receptor, stem cell factor, and mast cells. *Am. J. Pathol.* **142**, 965-974.

Gattuso JM, Hoyle CH, Milner P, Kamm MA, Burnstock G (1996) Enteric innervation in idiopathic megarectum and megacolon. *Int. J. Colorect. Dis.* **11**, 264-271.

Gattuso JM, Kamm MA, Talbot IC (1997) Pathology of idiopathic megarectum and megacolon. *Gut* **41**, 252-257.

Gayle JM, Blikslager AT, Bowman KF (2000) Mesenteric rents as a source of small intestinal strangulation in horses: 15 cases (1990-1997). *J. Am. vet. med. Ass.* **216**, 1446-1449.

Greet TR (1992) Ileal intussusception in 16 young thoroughbreds. *Equine vet. J.* **24**, 81-83.

Grider JR (1994) CGRP as a transmitter in the sensory pathway mediating peristaltic reflex. *Am. J. Phys.* **266,** G1139-G1145.

Gunn M (1968) Histological and histochemical observations on the myenteric and submucous plexuses of mammals. *J. Anat.* **102**, 221-239.

Hackett RP (1983) Nonstrangulated colonic displacement in horses. *J. Am. Vet. Med. Assoc.* **182**, 235-240.

Hagger R, Gharaie S, Finlayson C, Kumar D (1998a) Regional and transmural density of interstitial cells of Cajal in human colon and rectum. *Am. J. Phys.* **275**, G1309-G1316.

Hagger R, Gharaie S, Finlayson C, Kumar D (1998b) Distribution of the interstitial cells of Cajal in the human anorectum. *J. Auton. Nerv. Syst.* **73**, 75-79.

Hagger R, Finlayson C, Kahn F, De Oliveira R, Chimelli L, Kumar D (2000) A deficiency of interstitial cells of Cajal in Chagasic megacolon. *J. Auton. Nerv. Syst.* **80**, 108-111.

Hahn CN, Mayhew IG (2000) Phenylephrine eyedrops as a diagnostic test in equine grass sickness. *Vet. Rec.* **147**, 603-606.

Hahn CN, Mayhew IG, de Lahunta A (2001) Central neuropathology of equine grass sickness. *Acta Neuropathol.* **102**, 153-159.

Hara Y, Kubota M, Szurszewski JH (1986) Electrophysiology of smooth muscle of the
small intestine of some mammals. *J. Phys.* **372**, 501-520.

Hart C, Schochetman G, Spira T, Lifson A, Moore J, Galphin J, Sninsky J (1988) Direct detection of HIV RNA expression in seropositive subjects. *Lancet* **8611**, 596-599.

He CL, Burgart L, Wang L, Pemberton J, Young-Fadok T, Szurszewski J, Farrugia G (2000) Decreased interstitial cell of Cajal volume in patients with slow-transit constipation. *Gastroenterology* **118**, 14-21.

Heid CA, Stevens J, Livak KJ, Williams PM (1996) Real time quantitative PCR. *Genome Res.* **6**, 986-994.

Herdt T (1992) Chapter IV: Gastrointestinal Physiology/Metabolism. In: *Textbook of Veterinary Physiology.* Ed: Cunningham JG, WB Saunders Company, Independence Square West, Philadelphia, PA 19106, USA, pp251-363.

Hershko A, Ciechanover A (1992) The ubiquitin system for protein degradation. *Annu. Rev. Biochem.* **61**, 761-807.

Hillyer MH, Taylor FGR, French NP (2001) A cross-sectional study of colic in horses on Thoroughbred training premises in the British isles in 1997. *Equine vet. J.* **33**, 380-385.

Hillyer MH, Taylor FGR, Proudman CJ, Edwards GB, Smith JE, French NP (2002) Case control study to identify risk factors for simple colonic obstruction and distension colic in horses. *Equine vet. J.* **34**, 455-463.

Hinnebusch AG (1994) Translational control of *GCN4*: an *in vivo* barometer of initiation-factor activity. *Tech. Biochem. Sci.* **19**, 409-414.

Horiguchi K, Semple GSA, Sanders KM, Ward SM (2001) Distribution of pacemaker function through the tunica muscularis of the canine antrum. *J. Phys.* **537**, 237-250.

Horowitz B, Ward SM, Sanders KM (1999) Cellular and molecular basis for electrical rhythmicity in gastrointestinal muscles. *Annu. Rev. Phys.* **61**, 19-43.

Huang E, Nocka K, Beier DR, Chu TY, Buck J, Lahm HW, Wellner D, Leder P, Besmer P (1990) The hematopoietic growth factor KL is encoded by the *Sl* locus and is the ligand of the c-*kit* receptor, the gene product of the W locus. *Cell* **63**, 167-174.

Hudson NPH, Pearson GT, Kitamura N, Mayhew IG (1999) An immunohistochemical study of interstitial cells of Cajal in the equine gastrointestinal tract. *Res. vet. Sci.* **66**, 265-271.

Hudson JM, Cohen ND, Gibbs PG, Thompson JA (2001a) Feeding practices associated with colic in horses. *J. Am. vet. med. Ass.* **219**, 1419-1425.

Hudson NPH, Pearson GT, Kitamura N, Mayhew IG (2001b) A reduction in interstitial cells of Cajal in horses with equine dysautonomia (grass sickness). *Autonom. Neurosci.* **92**, 37-44.

Hudson NPH, Pearson GT, Mayhew IG (2001c) In vitro microelectrode study of the electrical properties of smooth muscle in equine ileum. *Vet. Rec.* **149**, 707-711.

Hudson NPH, Pearson GT, Mayhew IG (2002) Presence of in vitro electrical activity in the ileum of horses with enteric nervous system pathology: equine dysautonomia (grass sickness). *Autonom. neurosci.* **99**, 119-126.

Huizinga JD, Berezin I, Daniel EE, Chow E (1990) Inhibitory innervation of colonic smooth muscle cells and interstitial cells of Cajal. *Can. J. Phys.* **68**, 447-454.

Huizinga JD, Thuneberg L, Kluppel M, Malusz J, Mikkelsen HB, Bernstein A (1995) W/kit gene required for interstitial cells of Cajal and for intestinal pacemaker activity. *Nature* **373**, 347-349.

Hunter LC, Miller JK, Poxton IR (1999) The association of *Clostridium botulinum* type C with equine grass sickness: a toxicoinfection? *Equine Vet. J.* **31**, 429-499.

Irwin DA (1931) The anatomy of Auerbach's plexus. *Am. J. Anat.* **49**, 141-166.

Isozaki K, Hirota S, Nakama A, Miyagawa J-I, Shinomura Y, Xu Z, Nomura S, Kitamura Y (1995) Disturbed intestinal movement, bile reflux to the stomach, and deficiency of *c-kit*-expressing cells in ws/ws mutant rats. *Gastroenterology* **109**, 456-464.

Isozaki K, Hirota S, Miyagawa J, Taniguchi M, Shinomura Y, Matsuzawa Y (1997) Deficiency of c-kit+ cells in patients with a myopathic form of chronic idiopathic intestinal pseudo-obstruction. *Am. J. Gastroenterol.* **92**, 332-334.

Izushi K, Fujiwara Y, Tasaka K (1992) Identification of vimentin in rat peritoneal mast cells and its phosphorylation in association with histamine release. *Immunopharm.* **23**, 153-161.

Johnston JK, Freeman DE (1997) Diseases and surgery of the large colon. *Vet. Clin. North Am.* **13**, 317-340.

Kaneene JB, Miller RA, Ross WA, Gallagher K, Marteniuk J, Rook J (1997) Risk factors for colic in the Michigan (USA) equine population. *Prev. vet. Med.* **30**, 23-36.

Keef KD, Murray DC, Sanders KM, Smith TK (1997) Basal release of nitric oxide induces an oscillatory motor pattern in canine colon. *J. Phys.* **499**, 773-786.

Kenny SE, Connell MG, Rintala RJ, Vaillant C, Edgar DH, Lloyd DA (1998a) Abnormal colonic interstitial cells of Cajal in children with anorectal malformations. *J. ped. Surg.* **33**, 130-132.

Kenny SE, Vanderwinden JM, Rintala RJ, Connell MG, Lloyd DA, Vanderhaegen JJ, De Laet MH (1998b) Delayed maturation of the interstitial cells of Cajal: A new diagnosis for transient neonatal pseudoobstruction. Report of two cases. *J. ped. Surg.* **33**, 94-98.

Kenny SE, Connell G, Woodward MN, Lloyd DA, Gosden CM, Edgar DH, Vaillant C (1999) Ontogeny of the interstitial cells of Cajal in the human intestine. *J. ped. Surg.* **34**, 1241-1247.

Kerr JF, Wyllie AH, Currie AR (1972) Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br. J. Cancer* **26**, 239-257.

Kim YC, Koh SD, Sanders KM (2002) Voltage-dependent inward currents of interstitial cells of Cajal from murine colon and small intestine. *J. Phys.* **541**, 797-810.

Kitamura N, Pearson GTP, Cottrell DF, Hudson NPH, McGorum BC, Doxey DL (1998) Immunohistochemical study of intestinal neurons in horses with and without grass sickness. *Proceedings Equine Grass Sickness Fund Symposium, Equine Grass Sickness Fund 10th Anniversary Symposium 10th October 1998, Moredun Foundation, Edinburgh.*

Klüppel M, Huizinga JD, Malysz J, Bernstein A (1998) Developmental origin and *kit*-dependent development of the interstitial cells of Cajal in the mammalian small intestine. *Dev. Dyn.* **21**, 60-71.

Knowles CH, Martin JE (2000) Slow transit constipation: A model of human gut dysmotility. Review of possible aetiologies. *Neurogastroent. Motil.* **12**, 181-196.

Kobilo T, Szurszewski JH, Farrugia G, Hanani M (2003) Coupling and innervation patterns of interstitial cells of Cajal in the deep muscular plexus of the guinea-pig. *Neurogastroent. Motil.* **15**, 635-641.

Koh SD, Sanders KM, Ward SM (1998) Spontaneous electrical rhythmicity in cultured interstitial cells of Cajal from the murine small intestine. *J. Phys.* **513**, 203-213.

Koh SD, Jun JY, Kim TW, Sanders KM (2002) A Ca^{2+}-inhibited non-selective cation conductance contributes to pacemaker currents in mouse interstitial cells of Cajal. *J. Phys.* **540**, 803-814.

Koh SD, Ward SM, Ördög T, Sanders KM, Horowitz B (2003) Conductances responsible for slow wave generation and propagation in interstitial cells of Cajal. *Curr. Opin. Pharm.* **3**, 579-582.

König A, Corbacioglu S, Ballmaier M, Welte K (1997) Downregulation of c-*kit* expression in human endothelial cells by inflammatory stimuli. *Blood* **90**, 148-155.

Lammie A, Drobnjak M, Gerrald W, Saad A, Cote R, Cordon-Cardo C (1994) Expression of c-kit and ligand proteins in human tissues. *J. Histochem. Cytochem.* **42**, 1417-1425.

Langer JC, Berezin I, Daniel EE (1995) Hypertrophic pyloric stenosis: Ultrastructural abnormalities of enteric nerves and the interstitial cells of Cajal. *J. ped. Surg.* **30**, 1535-1543.

Lecoin L, Gabella G, LeDouarin N (1996) Origin of the *c-kit*-positive interstitial cells in the avian bowel. *Development* **122**, 725-733.

Lee HK, Sanders KM (1993) Comparison of ionic currents from interstitial cells and smooth muscle cells of canine colon. *J. Phys.* **460**, 135-152.

Lester GD, Merritt AM, Neuwirth L, Vetro-Widenhouse T, Steible C, Rice B, Mauragis DF, Madison JB (1998) Myoelectric activity of the ileum, cecum, and right ventral colon, and cecal emptying of radiolabeled markers in clinically normal ponies. *Am. J. vet. Res.* **59**, 313-319.

Little D, Blikslager AT (2002) Factors associated with development of ileal impaction in horses with surgical colic: 78 cases (1986-2000). *Equine vet. J.* **34**, 464-468.

Liu LWC, Farraway L, Berezin I (1998) Interstitial cells of Cajal: mediators of communication between circular and longitudinal muscle layers of canine colon. *Cell Tiss. Res.* **294**, 69-79.

Lopes MAF, Pfeiffer CJ (2000) Functional morphology of the equine pelvic flexure and its role in disease. A review. *Histol. Histopathol.* **15**, 983-991.

Love S (1992) The role of equine strongyles in the pathogenesis of colic and current options for prophylaxis. *Equine vet. J. Suppl.* **13**, 5-9.

Love S, Hillyer MH, Murphy DM, Reid SWJ (1994) Historical and clinical data from 39 cases of colonic (pelvic flexure) impaction. *Proc. 5th Equine Colic Symp.* Georgia, USA, pp36-37.

Lowe JE, Sellers AF, Brondum J (1980) Equine pelvic flexure impaction. A model used to evaluate motor events and compare drug response. *Cornell Vet.* **70**, 401-412.

Lu G, Qian X, Berezin I, Telford GL, Huizinga JD, Sarna SK (1997) Inflammation modulates in vitro colonic myoelectric and contractile activity and interstitial cells of Cajal. *Am. J. Phys.* **273**, G1233-G1245.

Maeda H, Yamagata A, Nishikawa S, Yoshigana K, Kobayashi S, Nishi K, Nishikawa S (1992) Requirement of *c-kit* for development of intestinal pacemaker system. *Development* **116**, 369-375.

Mair TS, Hillyer MH (1997a) Chronic colic in the mature horse: A retrospective review of 106 cases. *Equine vet. J.* **29**, 415-420.

Mair TS, Hillyer MH (1997b) Recurrent colic in the mature horse: A retrospective review of 58 cases. *Equine vet. J.* **29**, 421-424.

Mair TS, Sutton DG, Love S (2000) Caecocaecal and caecocolic intussusceptions associated with larval cyathostomosis in four young horses. *Equine vet. J.* **32**, 77-80.

Malone ED, Brown DR, Trent AM, Turner TA (1996) Influence of adrenergic and cholinergic mediators on the equine jejunum in vitro. *Am. J. vet. Res.* **57**, 884-890.

Malone ED, Kannan MS, Brown DR, Turner TA, Trent AM (1999) Adrenergic, cholinergic, and nonadrenergic-noncholinergic intrinsic innervation of the jejunum in horses. *Am. J. vet. Res.* **60**, 898-904.

McCarthy HE, French NP, Edwards GB, Poxton IR, Kelly DF, Payne-Johnson CE, Miller K, Proudman CJ (2004) Equine grass sickness is associated with low antibody levels to Clostridium botulinum: a matched case-control study. *Equine Vet. J.* **36**, 123-129.

Merritt AM, Campbell-Thompson ML, Lowrey S (1989a) Effect of xylazine treatment on equine proximal gastrointestinal tract myoelectrical activity. *Am. J. vet. Res.* **50**, 945-949.

Merritt AM, Campbell-Thompson ML, Lowrey S (1989b) Effect of butorphanol on equine antroduodenal motility. *Equine vet. J. Suppl.* **7**, 21-23.

Merritt AM, Panzer RB, Lester GD, Burrow JA (1995) Equine pelvic flexure myoelectric activity during fed and fasted states. *Am. J. Phys.* **269**, G262-G268.

Merritt AM, Burrow JA, Harless CS (1998) Effect of xylazine, detomidine, and a combination of xylazine and butorphanol on equine duodenal motility. *Am. J. vet. Res.* **59**, 619-623.

Metallinos DL, Bowling AT, Rine J (1998) A missense mutation in the endothelin-B receptor gene is associated with Lethal White Foal Syndrome: an equine version of Hirschsprung disease. *Mamm. Genome* **9**, 426-431.

Mikkelsen HB, Malysz J, Huizinga JD, Thuneberg L (1998) Action potential generation, Kit receptor immunohistochemistry and morphology of *steel-Dickie (Sl/Sl^d)* mutant mouse small intestine. *Neurogastroenterol. Motil.* **10**, 11-26.

Milne EM, Woodman MP, Doxey DL (1994) Use of clinical measurements to predict outcome in chronic cases of grass sickness (equine dysautonomia). *Vet. Rec.* **143**, 438-440.

Milne EM (1997) Clinical aspects and treatment of grass sickness in the UK. *Proceedings of the 1st International Workshop on Grass Sickness, EMND and related disorders*, Bern, 26-27th October 1995. *Equine Vet. J.*, June 1997, 1-3.

Moncada S, Palmer RM, Higgs EA (1991) Nitric oxide: Physiology, pathophysiology, and pharmacology. *Pharm. Rev.* **43**, 109-142.

Murray A, Cottrell DF, Woodman MP (1994) Cholinergic activity of intestinal muscle *in vitro* taken from horses with and without equine grass sickness. *Vet. Res. Comm.* **18**, 199-207.

Nieto JE, Snyder JR, Kollias-Baker C, Stanley S (2000) In vitro effects of 5-hydroxytryptamine and cisapride on the circular smooth muscle of the jejunum of horses. *Am. J. vet. Res.* **61**, 1561-1565.

Nocka K, Tan JC, Chiu E, Chu TY, Ray P, Traktman P, Besmer P (1990) Molecular bases of dominant negative and loss of function mutations at the murine c-*kit*/white spotting locus: W^{37}, W^{V}, W^{41} and *W. EMBO* **9**, 1805-1813.

Obel A-L (1955) Studies on grass disease. The morphological picture with special reference to the vegetative nervous system. *J. comp. Path.* **65**, 334-349.

Oh J-T, Han A, Yang W-I, Han SJ, Choi SH, Hwang EH (2002) Morphometric evaluation of PGP9.5 and NCAM expressing nerve fibers in colonic muscle of patients with Hirschprung's disease. *Yonsei med. J.* **43**, 31-36.

Ossovskaya VS, Bunnett NW (2004) Protease-activated receptors contribute to physiology and disease. *Phys. Rev.* **84**, 579-621.

Owen Rh ap Rh, Kelly DF (2003) Complete clinical recovery of a pony with histologically confirmed chronic grass sickness. *Vet. Rec.* **153**, 597-598.

Palmer JE (1985) Gastrointestinal diseases of foals. *Vet. Clin. North Am.* **5**, 151-168.

Parks AH, Doran RE, White NA, Baxter GM (1989) Ileal impaction in the horse: 75 cases. *Cornell Vet.* **79**, 83-91.

Pearson GT (1994) Structural organization and neuropeptide distributions in the equine enteric nervous system: an immunohistochemical study using whole-mount preparations from the small intestine. *Cell Tissue Res.* **276**, 523-534.

Platt H (1986) Chronic inflammatory and lymphoproliferative lesions of the equine small intestine. *J. Comp. Pathol.* **96**, 671-684.

Plujà L, Albertí E, Fernández E, Mikkelsen HM, Thuneberg L, Jiménez M (2001) Evidence supporting presence of two pacemakers in rat colon. *Am. J. Phys*, **281**, G255-G266.

Pogson DM, Doxey DL, Gilmour JS, Milne EM, Chisholm HK (1992) Autonomic neurone degeneration in equine dysautonomia (grass sickness). *J. comp. Path.* **107**, 271-283.

Prince D, Cocoran BM, Mayhew IG (2003) Changes in nasal mucosal innervation in horses with grass sickness. *Equine vet. J.* **35**, 60-66.

Proudman CJ (1991) A two year, prospective survey of equine colic in general practice. *Equine vet. J.* **24**, 90-93.

Proudman CJ, French NP, Trees AJ (1998) Tapeworm infection is a significant risk factor for spasmodic colic and ileal impaction colic in the horse. *Equine vet. J.* **30**, 194-199.

Publicover NG, Hammond EM, Sanders KM (1993) Amplification of nitric oxide signalling by interstitial cells isolated from canine colon. *Proc. Nat. Acad. Sci.* **90**, 2087-2091.

Rae MG, Fleming N, McGregor DB, Sanders KM, Keef KD (1998) Control of motility patterns in the human colonic circular muscle layer by pacemaker activity. *J. Phys.* **510**, 309-320.

Rakestraw PC, Snyder JR, Woliner MJ, Sanders KM, Shuttleworth WR (1996) Involvement of nitric oxide in inhibitory neuromuscular transmission in equine jejunum. *Am. J. vet. Res.* **57**, 1206-1213.

Rakestraw PC, Snyder JR, Sanders KM, Shuttleworth WR (2000) Intracellular microelectrode recording to characterize inhibitory neuromuscular transmission in jejunum of horses. *Am. J. vet. Res.* **61**, 362-368.

Re G, Belloli C, Badino P, Arioli F, Novelli A, Giardi C, Beretta C (1997) Identification of ß-adrenenergic receptor subtypes mediating relaxation in isolated equine ileum. *Am. J. vet. Res.* **58**, 621-625.

Re MG, Fleming N, McGregor DB, Sanders KM, Keef KD (1998) Control of motility patterns in the human colonic circular muscle layer by pacemaker activity. *J. Phys.* **510**, 309-320.

Reeves MJ, Salman MD, Smith G (1996) Risk factors for equine acute abdominal disease (colic): Results from a multi-centre case-control study. *Prev. vet. Med.* **26**, 285-301.

Reid SW, Mair TS, Love S (1995) Epidemiological risk factors associated with a diagnosis of clinical cyathostomiasis in the horse. *Equine vet. J.* **27**, 127-130.

Rich A, Miller SM, Gibbons SJ, Malysz J, Szurszewski JH, Farrugia G (2003) Local presentation of Steel factor increases expression of c-kit immunoreactive interstitial cells of Cajal in culture. *Am. J. Phys.* **284**, G313-G320.

Roger T, Ruckebusch Y (1987) Pharmacological modulation of postprandial colonic motor activity in the pony. *J. vet. Pharmacol. Therap.* **10**, 273-282.

Romans P, Tu Z, Ke Z, Hagedorn HH (1995) Analysis of a vitellogenin gene of the mosquito, Aedes aegypti and comparisons to vitellogenins from other organisms. *Insect Biochem Mol Biol.* **8**, 939-58.

Rossdale PD, Ousey JC, Cottrill CM, Chavatte P, Allen WR, McGladdery AJ (1991)
Effects of placental pathology on maternal plasma progestagen and mammary secretion calcium concentrations and on neonatal adrenocortical function in the horse. *J. Reprod. Fertil. Suppl.* **44**, 579-590.

Rossdale PD (1993) Clinical view of disturbances in equine foetal maturation. *Equine vet. J. Suppl.* **14**, 3-7.

Rottapel R, Reedijk M, Williams DE, Lyman SD, Anderson DM, Pawson T, Bernstein A (1991) The *Steel/W* transduction pathway: Kit autophoshorylation and its association with a unique subset of cytoplasmic signaling proteins is induced by the Steel factor. *Mol. Cell. Biol.* **11**, 3043-3051.

Roussel AJ, Hooper RN, Cohen ND, Bye AD, Hicks RJ, Bohl TW (2000) Prokinetic effects of erythromycin on the ileum, cecum, and pelvic flexure of horses during the postoperative period. *Am. J. Vet. Res.* **61**, 420-424.

Rowe EL, White NA, Buechner-Maxwell V, Robertson JL, Ward DL (2003) Detection of apoptotic cells in intestines from horses with and without gastrointestinal tract disease. *Am. J. Vet. Res.* **64**, 982-988.

Ruckebusch Y, Fioramonti J (1980) Colonic myoelectrical spiking activity: major patterns and significance in six different species. *Zbl. Vet. Med. A.* **27**, 1-8.

Ruckebusch Y (1981) Motor functions of the intestine. *Adv. vet. Sci. comp. Med.* **25**, 345-369.

Ruckebusch Y, Roger T (1988) Prokinetic effects of cisapride, naloxone and parasympathetic stimulation at the equine ileo-caeco-colonic junction. *J. vet. Pharmacol. Therap.* **11**, 322-329.

Rudin CM, Thompson CB (1997) Apoptosis and disease: regulation and clinical relevance of programmed cell death. *Annu. Rev. Med.* **48**, 267-281.

Rumessen JJ, Mikkelsen HB, Qvortrup K, Thuneberg L (1993) Ultrastructure of interstitial cells of Cajal in circular muscle of human small intestine. *Gastroenterology*, **104**, 343-350.

Rumessen JJ (1996) Ultrastructure of interstitial cells of Cajal at the colonic submucosal border in patients with ulcerative colitis. *Gastroenterology* **111**, 1447-1455.

Russell ES (1979) hereditary anemias of the mouse: a review for geneticists. *Adv. Gen.* **20**, 357-459.

Saiki RK, Scharf S, Faloona F, Mullins KB, Horn GT, Erlich HA, Arnheim N (1985) Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. *Science*, **230**, 1350-1354.

Saiki RK, Chang CA, Levenson CH, Warren TC, Boehm CD, Kazazian HH Jr, Erlich HA (1988) Diagnosis of sickle cell anemia and beta-thalassemia with enzymatically amplified DNA and nonradioactive allele-specific oligonucleotide probes. *N. Engl. J. Med.* **319**, 537-541.

Sams VR, Bobrow LG, Happerfield L, Keeling J (1992) Evaluation of PGP9.5 in the diagnosis of Hirschprung's disease. *J. Path.* **168**, 55-58.

Sanders KM, Smith TK (1986a) Enteric neural regulation of slow waves in circular muscle of the canine proximal colon. *J. Phys.* **377**, 297-313.

Sanders KM, Smith TK (1986b) Motorneurones of submucous plexus regulate electrical activity of the circular muscle of the canine proximal colon. *J. Phys.* **380**, 293-310.

Sanders KM, Stevens R, Burke EP, Ward SM (1990) Slow waves actively propagate at submucosal surface of circular layer in canine colon. *Am. J. Physiol.* **259**, G258-G263.

Sanders KM, Ward SM (1992) Nitric oxide as a mediator of nonadrenergic noncholinergic neurotransmission. *Am. J. Phys.* **262**, G379-G392.

Sanders KM (1996) A case for interstitial cells of Cajal as pacemakers and mediators of neurotransmission in the gastrointestinal tract. *Gastroenterology* **111**, 492-515.

Sanders KM, Ördög T, Koh SD, Torihashi S, Ward SM (1999) Development and plasticity of interstitial cells of Cajal. *Neurogastroenterol. Motil.* **11**, 311-338.

Sanders KM, Koh SD, Ördög T, Ward SM (2004) Ionic conductances involved in generation and propagation of electrical slow waves in phasic gastrointestinal muscles. *Neurogastroenterol. Motil. Suppl. 1* **16**, 100-106.

Santschi EM, Purdy AK, Valberg SJ, Vrotsos PD, Kaese H, Mickelson JR (1998) Endothelin receptor B polymorphism associated with lethal white foal syndrome in horses. *Mamm. Genome* **9**, 306-309.

Sarna S, Northcott P, Belbeck L (1982) Mechanism of cycling of migrating myoelectric complexes: effect of morphine. *Am. J. Physiol.* **242**, G588-G595.

Sarna SK, Condon R, Cowles V (1984) Colonic migrating and nonmigrating motor complexes in dogs. *Am. J. Physiol.* **246**, G355-G360.

Sarna SK (1986) Myoelectric correlates of colonic motor complexes and contractile activity. *Am. J. Physiol.* **250**, G213-G220.

Scheuermann DW, Stach W, Timmermanns J-P, Adriansen D, De Groodt-Lasseel MHA (1989) Neuron-specific enolase and S-100 protein immunohistochemistry for defining the structure and topographical relationship of the different enteric nerve plexuses in the small intestine of the pig. *Cell Tissue Res.* **256**, 65-75.

Scholes SFE, Vaillant C, Peacock P, Edwards GB, Kelly DF (1993) Diagnosis of grass sickness by ileal biopsy. *Vet. Rec.* **133**, 7-10.

Schusser GF, White NA (1997) Morphologic and quantitative evaluation of the myenteric plexus and neurons in the large colon of horses. *J. Am. vet. med. Ass.* **210**, 928-934.

Schusser GF, Scheidemann W, Huskamp B (2000) Muscle thickness and neuron density in the caecum of horses with chronic recurrent caecal impaction. *Equine vet. J. Suppl.* **32**, 69-73.

Scott EA, Heidel JR, Snyder SP, Ramirez S, Whitler WA (1999) Inflammatory bowel disease in horses: 11 cases (1988-1998). *J. Am. vet. med. Ass.* **214**, 1527-1530.

Sellers AF, Lowe JE, Brondum J (1979) Motor events in the equine large colon. *Am. J. Phys.* **237**, E457-E464.

Sellers AF, Lowe JE, Drost CJ, Rendano VT, Georgi JR, Roberts MC (1982a) Retropulsion-propulsion in equine large colon. *Am. J. vet. Res.* **43**, 390-396.

Sellers AF, Lowe JE, Rendano VT, Drost CJ (1982b) The reservoir function of the equine cecum and ventral large colon- its relation to chronic non-surgical obstructive disease with colic. *Cornell Vet.* **72**, 233-241.

Sellers AF, Lowe JE, Rendano VT (1984) Equine colonic motility: Interactions among wall motion, propulsion, and fluid flow. *Am. J. vet. Res.* **45**, 357-360.

Sembrat RF (1975) The acute abdomen in the horse: Epidemiological considerations. *Arch. Am. Coll. Vet. Surg.* **4**, 34-39.

Sheik MS, Fornace Jr AJ (1999) Regulation of translation initiation following stress. *Oncogene* **18**, 6121-6128.

Smith TK, Reed JB, Sanders KM (1987a) Origin and propagation of electrical slow waves in circular muscle of canine proximal colon. *Am. J. Physiol.* **252**, C215-C224.

Smith TK, Reed JB, Sanders KM (1987b) Interaction of two electrical pacemakers in muscularis of canine proximal colon. *Am. J. Physiol.* **252**, C290-C299.

Sonea IM, Wilson DV, Bowker WRM, Robinson NE (1997) Tachykinin receptors in the equine pelvic flexure. *Equine vet. J.* **29**, 306-312.

Spencer NJ, Sanders KM, Smith TK (2003) Migrating motor complexes do not require electrical slow waves in the mouse small intestine. *J. Phys.* **553**, 881-893.

Staden R, Beal KF, Bonfield JK (2000) The Staden package, 1998. *Methods Mol. Biol.* **132**,115-130.

Steinert PM, Roop DR (1988) Molecular and cellular biology of intermediate filaments. *Annu. Rev. Biochem.* **57**, 593-625.

Stürzenbaum SR, Kille P (2001) Control genes in quantitative molecular biological techniques: the variability of invariance. *Comp. Biochem. Phys.* **130**, 281-289.

Suprenant A (1994) Control of the gastrointestinal tract by enteric neurons. *Annu. Rev. Physiol.* **56**, 117-140.

Suzuki N, Prosser CL, Dahms V (1986) Boundary cells between longitudinal and circular muscle layers: essential for electrical slow waves in cat intestine. *Am. J. Phys.* **250**, G287-G294.

Suzuki N, Takano H, Yamamoto Y, Komuro T, Saito M, Kato K, Mikoshiba K (2000) Properties of gastric smooth muscles obtained from mice which lack inositol triphosphate receptor. *J. Phys.* **525**, 105-111.

Szurszewski JH (1969) A migrating electric complex of canine small intestine. *Am. J. Physiol.* **217**, 1757-1763.

Taxi J (1952) Cellules de Schwann et "cellules interstitielles de Cajal" au niveau des plexus nerveux de la musculeuse intestinale du cobaye: retour aux definitions. *Arch. Anat. Microsc. Morphol. Exp.* **41**, 281-304.

Taxi J (1965) Contribution á l'étude des connexions des neurones moteurs du systeme nerveux sutonome. *Ann. Sci. Nat. Zool. Biol. Anim.* 7, 413-674.

Taylor I, Duthie HL, Smallwood R, Linkens D (1975) Large bowel myoelectrical activity in man. *Gut* **16**, 808-814.

Taylor AB, Kreulen D, Prosser CL (1977) Electron microscopy of the connective tissues between longitudinal and circular muscle of small intestine of cat. *Am. J. Anat.* **150**, 427-442.

Thompson CB (1995) Apoptosis in the pathogenesis and treatment of disease. *Science* **267**, 1456-1462.

Thomsen L, Pearson GT, Hviid Larsen E, Skadhauge E (1997) Electrophysiological properties of neurones in the internal and external submucosal plexuses of newborn pig small intestine. *J. Phys.* **498**, 773-785.

Thomsen L, Robinson TL, Lee JCF, Farraway LA, Hughes MJG, Andrews DW, Huizinga JD (1998) Interstitial cells of Cajal generate a rhythmic pacemaker current. *Nature Med.* **4**, 848-851.

Thuneberg L (1982) Interstitial cells of Cajal: Intestinal pacemaker cells. *Adv. Anat. Embryol. Cell Biol.* **71**, 1-130.

Timmermanns J-P, Scheuermann DW, Stach W, Adriaensen D, De Groodt-Lasseel MHA (1990) Distinct distribution of CGRP-, enkephalin-, galanin-, neuromedin U-, neuropeptide Y-, somatostatin-, substance P-, VIP- and serotonin- containing neurons in the two submucosal ganglionic neural networks of the porcine small intestine. *Cell Tissue Res.* **260**, 367-379.

Tinker MK, White NA, Lessard P, Thatcher CD, Peltzer KD, Davis B, Carmel D (1997a) Prospective study of equine colic incidence and mortality. *Equine vet. J.* **29**, 448-453.

Tinker MK, White NA, Lessard P, Thatcher CD, Peltzer KD, Davis B, Carmel D (1997b) Prospective study of equine colic risk factors. *Equine vet. J.* **29**, 454-458.

Torihashi S, Kobayashi S, Gerthoffer WT, Sanders KM (1993) Interstitial cells in deep muscular plexus of canine small intestine may be specialized smooth muscle cells. *Am. J. Phys.* **265**, G638-G645.

Torihashi S, Gerthoffer WT, Kobayashi S, Sanders KM (1994) Identification and classification of interstitial cells in the canine proximal colon by ultrastructure and immunohistochemistry. *Histochemistry* **101**, 169-183.

Torihashi S, Ward SM, Nishikava S-I, Nishi K, Kobayashi S, Sanders KM (1995) C-kit-dependent development of interstitial cells and electrical activity in the murine gastrointestinal tract. *Cell Tissue Res.* **280**, 97-111.

Torihashi S, Ward SM, Sanders KM (1997) Development of c-kit-positive cells and the onset of electrical rhythmicity in murine small intestine. *Gastroenterology* **112**, 144-155.

Torihashi S, Nishi K, Tokutomi Y, Nishi T, Ward SM, Sanders KM (1999) Blockade of kit signaling induces transdifferentiation of interstitial cells of Cajal to a smooth muscle phenotype. *Gastroenterology* **117**, 140-148.

Traub-Dargatz JL, Kopral CA, Seitzinger AH, Garber LP, Forde K, White NA (2001) Estimate of the national incidence of and operation-level risk factors for colic among horses in the United States, spring 1998 to spring 1999. *J. Am. vet. med. Ass.* **219**, 67-71.

Uhlinger C (1990) Effects of three anthelmintic schedules on the incidence of colic in horses. *Equine vet. J.* **22**, 251-254.

Uzal FA, Robles CA (1997a) Clinical signs of mal seco, a grass sickness-like syndrome of horses. *Proceedings of the 1st International Workshop on Grass Sickness, EMND and related disorders*, Bern, 26-27th October 1995. *Equine Vet. J.*, June 1997, 7-8.

Uzal FA, Robles CA (1997b) *Post mortem* findings in "mal seco", a grass sickness-like syndrome of horses. *Proceedings of the 1st International Workshop on Grass Sickness, EMND and related disorders*, Bern, 26-27th October 1995. *Equine Vet. J.*, June 1997, 21-22.

Vaala WE (1994) Peripartum asphyxia. *Vet. Clin. North Am.* **10**, 187-218.

Vanderwinden J-M, Rumessen JJ (1999) Interstitial cells of Cajal in human gut and gastrointestinal disease. *Microsc. Res. Techn.* **47**, 344-360.

Van Hoogmoed LM, Rakestraw PC, Snyder JR, Harmon FA (2000) Evaluation of nitric oxide as an inhibitory neurotransmitter in the equine ventral colon. *Am. J. vet. Res.* **61**, 64-68.

Wang XY, Sanders KM, Ward SM (1999) Intimate relationship between interstitial cells of Cajal and enteric nerves in the guinea-pig small intestine. *Cell Tissue Res.* **295**, 247-256.

Wang XY, Sanders KM, Ward SM (2000) Relationship between interstitial cells of Cajal and enteric motor neurons in the murine proximal colon. *Cell Tissue Res.* **302**, 331-342.

Wang XY, Berezin I, Mikkelsen HB, Der T, Bercik P, Collins SM, Huizinga JD, Huizina JD (2002) Pathology of interstitial cells of Cajal in relation to inflammation revealed by ultrastructure but not immunohistochemistry. *Am. J. Path.* **160**, 1529-1540.

Ward SM, Sanders KM (1990) Pacemaker activity in septal structures of canine colonic circular muscle. *Am. J. Physiol.* **258**, G264-G273.

Ward SM, Keller R, Sanders KM (1991) Structure and organization of the electrical activity of the canine distal colon. *Am. J. Physiol.* **260**, G724-G735.

Ward SM, Sanders KM (1992) Dependence of electrical slow waves of canine colonic smooth muscle on calcium gradient. *J. Phys.* **455**, 307-319.

Ward SM, Burns AJ, Torihashi S, Sanders KM (1994) Mutation of the proto-oncogene *c-kit* blocks development of interstitial cells and electrical activity in the murine gastrointestinal tract. *J. Physiol.* **480**, 91-97.

Ward SM, Burns AJ, Torihashi S, Harney SC, Sanders KM (1995) Impaired development of interstitial cells and intestinal electrical rhythmicity in *steel* mutants. *Am. J. Phys.* **269**, C1577-C1585.

Ward SM, Harney SC, Bayguyinov JR, McLaren GJ, Sanders KM (1997) Development of electrical rhythmicity in the murine gastrointestinal tract is specifically encoded in the tunica muscularis. *J. Physiol.* **505**, 241-258.

Ward SM, Morris G, Reese L, Wang X-Y, Sanders KM (1998) Interstitial cells mediate enteric inhibitory neurotransmission in the lower esophageal and pyloric sphincters. *Gastroenterology* **115**, 314-329.

Ward SM, Ördög T, Koh SD, Bayguinov JR, Horowitz B, Epperson E, Shen L, Westphal H, Sanders KM (1999) Development of interstitial cells of Cajal and pacemaking in mice lacking enteric nerves. *Gastroenterology* **117**, 584-594.

Ward SM, Ördög T, Koh SD, Abu Baker S, Jun JY, Sanders KM (2000) Pacemaking in interstitial cells of Cajal depends upon calcium handling by endoplasmic reticulum and mitochondria. *J. Phys.* **525**, 355-361.

Ward SM, Sanders KM (2001) Physiology and pathophysiology of the interstitial cells of Cajal: From bench to bedside. I. Functional development and plasticity of interstitial cells of Cajal networks. *Am. J. Phys.* **281**, G602-G611.

Ward SM, Gershon MD, Keef K, Bayguinov YR, Nelson C, Sanders KM (2002) Interstitial cells of Cajal and electrical activity in ganglionic and aganglionic colons of mice. *Am. J. Physiol.* **283**, G445-G456.

Weiss R, Abel D, Scholtysik G, Straub R, Mevissen M (2002) 5-Hydroxytryptamine mediated contractions in isolated preparations of equine ileum and pelvic flexure: pharmacological characterization of a new 5-HT_4 agonist. *J. vet. Pharmacol. Therap.* **25**, 49-58.

Wester T, Eriksson L, Olsson Y, Olsen L (1999) Interstitial cells of Cajal in the human fetal small bowel as shown by c-kit immunohistochemistry. *Gut* **44**, 65-71.

White NA (1990) In: The Equine Acute Abdomen. Lea & Febiger, Philadelphia, pp49-61.

White NA, Dabareiner RM (1997) Treatment of impaction colics. *Vet. Clin. North Am.* **13**, 243-259.

Whitwell K (1997) Histopathology of grass sickness-comparative aspects of dysautonomia in various species (equine, feline, canine, leprids). *Proceedings of the 1st International Workshop on Grass Sickness, EMND and related disorders*, Bern, 26-27th October 1995. *Equine Vet. J.*, June 1997, 18-20.

Wilson JH, Cudd TA (1990) Section Three: Common Gastrointestinal Diseases. Ed: Koterba AM, Drummond WH, Kosch PC in Equine Clinical Neonatology. Lea and Febiger, Malvern, PA 19355-9725, USA, 412-430.

Williams DE, Eisenman J, Baird A, Rauch C, Van Ness K, March CJ, Park LS, Martin U, Mochizuki DY, Boswell HS (1990) Identification of a ligand for the c-*kit* proto-oncogene. *Cell* **63**, 167-174.

Willis AE (1999) Translational control of growth factor and proto-oncogene expression. *Biochem. Cell Biol.* **31**, 73-86.

Wu JJ, Rothman TP, Gershon MD (2000) Development of the interstitial cells of Cajal: origin, kit dependence and neuronal and nonneuronal sources of kit ligand. *J. Neurosci. Res.* **59**, 384-401.

Xue C, Pollock J, Schmidt HHHW, Ward SM, Sanders SM (1994) Expression of nitric oxide synthase by interstitial cells of the canine proximal colon. *J. autonom. nerv. Syst.* **49**, 1-14.

Yamataka A, Ohshiro K, Kobayashi H, Lane GL, Yamataka T, Fujiwara T, Sunagawa M, Miyano T (1998) Abnormal distribution of intestinal pacemaker (c-kit)-

positive cells in an infant with chronic idiopathic intestinal pseudoobstruction. *J. ped. Surg.* **33**, 859-862.

Yarden Y, Kuang W-J, Yang-Fen T, Coussens L, Munemitsu S, Dull TJ, Chen E, Schlessinger J, Francke U, Ullrich A (1987) Human proto-oncogene *c-kit:* A new cell surface receptor tyrosine kinase for an unidentified ligand. *EMBO* **6**, 3341-3351.

Yoo SY, Jung SH, Eom M, Kim IH, Han A (2002) Delayed maturation of interstitial cells of Cajal in meconium obstruction. *J. Pedriat. Surg.* **37**, 1758-1761.
Young HM, Ciampoli D, Southwell BR, Newgreen DF (1996) Origin of the interstitial cell of Cajal in the mouse intestine. *Dev. Biol.* **180**, 97-107.

Zamorano PL, Mahesh VB, Brann DW (1996) Quantitative RT-PCR for neuroendocrine studies. A minireview. *Neuroendocrinology* **63**, 397-407.

Zhong H, Simons JW (1999) Direct comparison of GAPDH, beta-actin, cyclophilin, and 28S rRNA as internal standards for quantifying RNA levels under hypoxia. *Biochem Biophys. Res. Commun.* **259**, 523-526.

Zsebo KM, Williams DA, Geissler EN, Broudy VC, Martin FH, Atkins HL, Hsu RY, Birkett NC, Okino KH, Murdock DC, Jacobsen FW, Langley KE, Smith KA, Takeshi T, Cattanach BM, Galli SJ, Suggs SV (1990) Stem cell factor is encoded at the *Sl* locus of the mouse and is the ligand for the c-*kit* tyrosine kinase receptor. *Cell* **63**, 213-224.

7 APPENDICES

APPENDIX 1

Phosphate-buffered saline

8.50g Sodium chloride
1.07g diSodium hydrogen orthophosphate anhydrous
0.34g Sodium dihydrogen orthophosphate-hydrate

The salt were dissolved in 1 litre of distilled water

APPENDIX 2

Loading buffer

0.025g Bromophenol blue 0.25% (w/v)
0.025g Xylene cyanol 0.25% (w/v)
1.5g Ficoll1 (type 400) 15% (w/v)

Make up to 10ml with distilled water

APPENDIX 3

TAE buffer (5x)

121g Tris(hydroxymethylaminomethane) (Tris)
28.55ml Glacial acetic acid
50ml (0.5M) Ethylendiamintetraacetic acid (EDTA)

Make up to 5 litres with distilled water

TAE buffer (1x)

1L of TAE buffer (5x) made up to 5L with 4L distilled water

APPENDIX 4

Real-time quantitative PCR data

File Quantification Report Window Help

Analysis
- (•) Fit Points
- ( ) Second Derivative Maximum

Baseline Adjustment
- ( ) None
- (•) Arithmetic
- ( ) Proportional
- ( ) Normalized

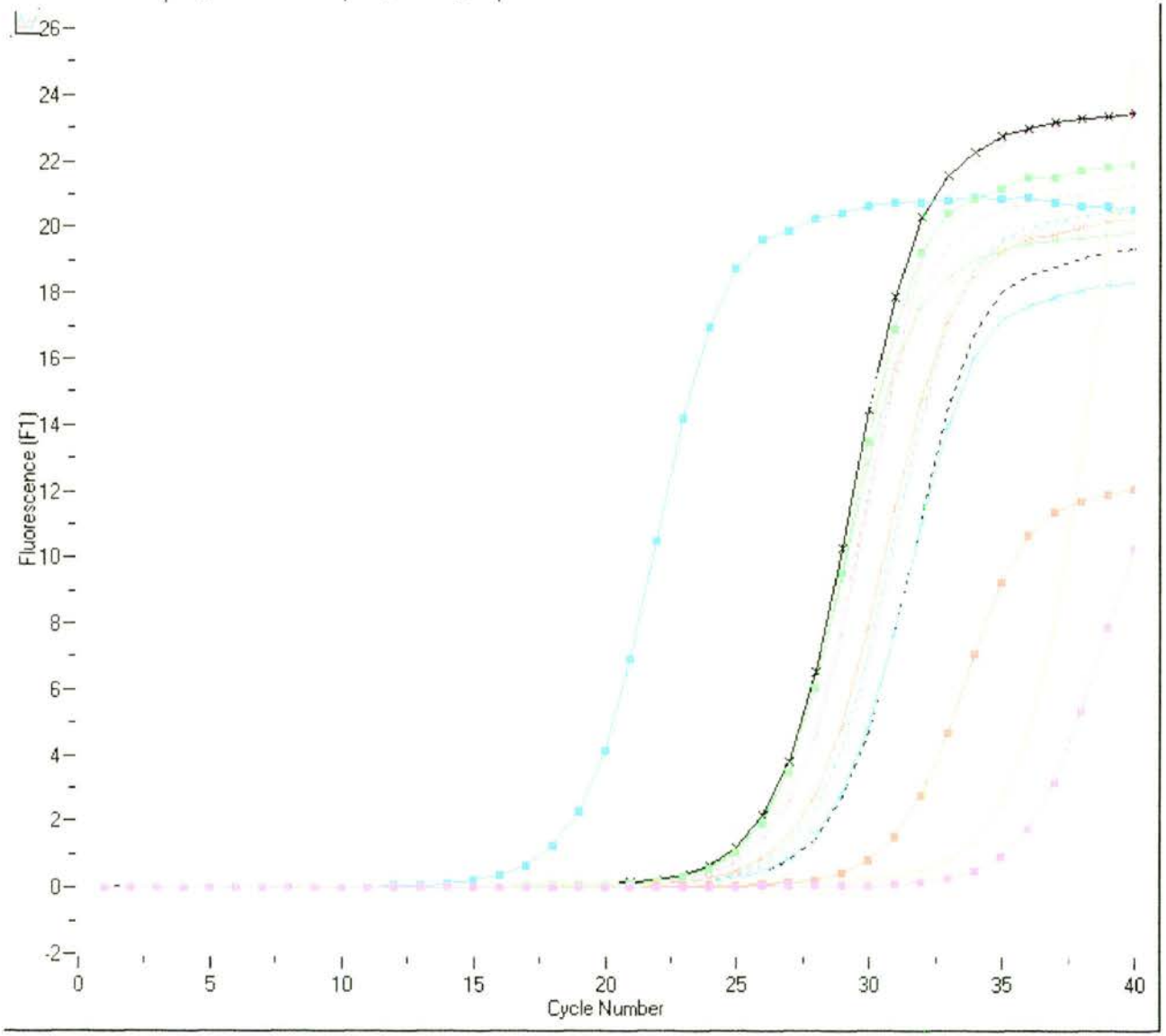
Number of Points: 2

☐ Show Fit Points

Analysis Notes: C-kit small intestine - PCR

P...	Name	Standard	Calculat...	Cro...
1	SSPCA		1.976E-08	23.68
2	03/488		2.504E-08	23.35
3	01/488		7.379E-08	21.88
4	KB		1.735E-08	23.86
5	02/56		1.190E-08	24.37
6	LAMB		1.012E-07	21.44
7	01/462		9.524E-08	21.53
10	03/381		5.156E-08	22.37
11	00/656		1.614E-08	23.95
12	02/90		8.538E-08	21.68
13	9/6/03		2.657E-08	23.27
14	03/435		5.641E-08	22.24
15	02/110		9.552E-08	21.52
16	02/97		3.101E-07	19.91
17	03/209		5.572E-10	28.56
18	27870		1.126E-08	24.45
19	C-KIT5	1.000E-05	1.253E-05	14.86
20	C-KIT7	1.000E-07	6.373E-08	22.08
21	C-KIT9	1.000E-09	1.253E-09	27.45
22	WATER		3.479E-11	32.35

Step 1: Baseline | Step 2: Noise Band | Step 3: Analysis



Start | Administrator | Data | Front | LCDA | 14:42

File Quantification Report Window Help

Analysis

Fit Points

Second Derivative Maximum

Baseline Adjustment

None

Arithmetic

Proportional

Normalized

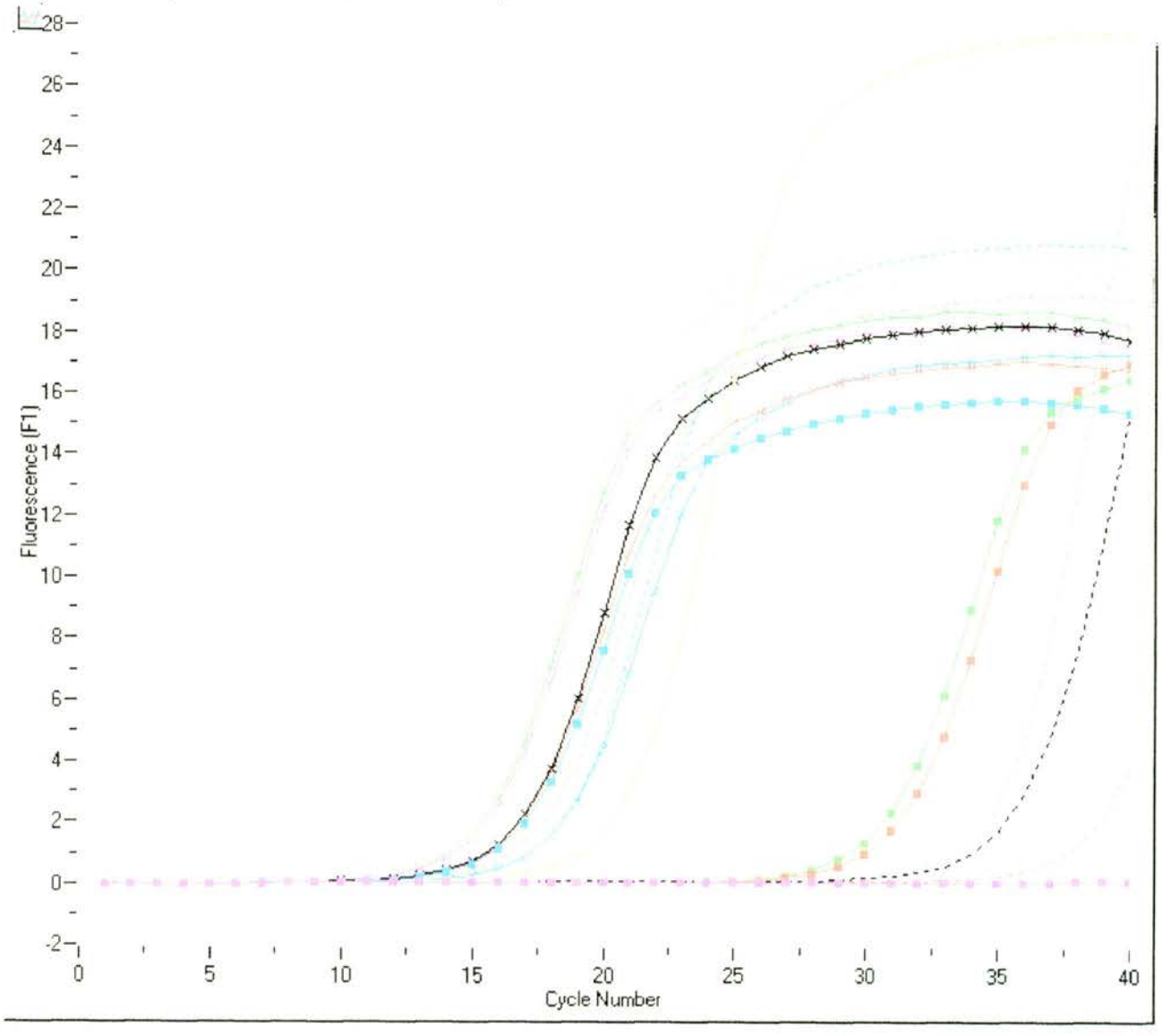
Number of Points: 2

Show Fit Points

Analysis Notes: β-actin small intestine -PCR

Step 1: Baseline | Step 2: Noise Band | Step 3: Analysis

P...	Name	Standard	Calculat...	Cro...
1	sspca old		3.651E-05	13.65
2	03/488		6.524E-05	12.90
3	01/488		1.333E-11	32.84
4	kb		3.226E-10	28.72
5	02/56		3.409E-10	28.64
6	lamb		2.594E-04	11.11
7	01/462		4.034E-04	10.54
8	03/381		1.218E-04	12.09
9	00/656		3.793E-05	13.60
10	02/90		3.639E-04	10.67
11	9/6/03		1.409E-04	11.90
12	03/435		3.480E-04	10.73
13	02/110		2.013E-04	11.44
14	02/97		9.640E-04	9.412
15	03/209		1.037E-05	15.28
16	27870		1.910E-05	14.49
17	bact4	1.000E-04	1.210E-04	12.10
18	bact8	1.000E-08	3.857E-09	25.50
19	bact9	1.000E-09	2.143E-09	26.26
20	water			



220

Start | Administrator | Front | LCDA | 14:45

LightCycler Data Analysis [Administrator] - [Quantification: CKIT FFNC, F1, AMPLIFICATION seg 3]

File Quantification Report Window Help

Analysis
- (•) Fit Points
- ( ) Second Derivative Maximum

Baseline Adjustment
- ( ) None
- (•) Arithmetic
- ( ) Proportional
- ( ) Normalized

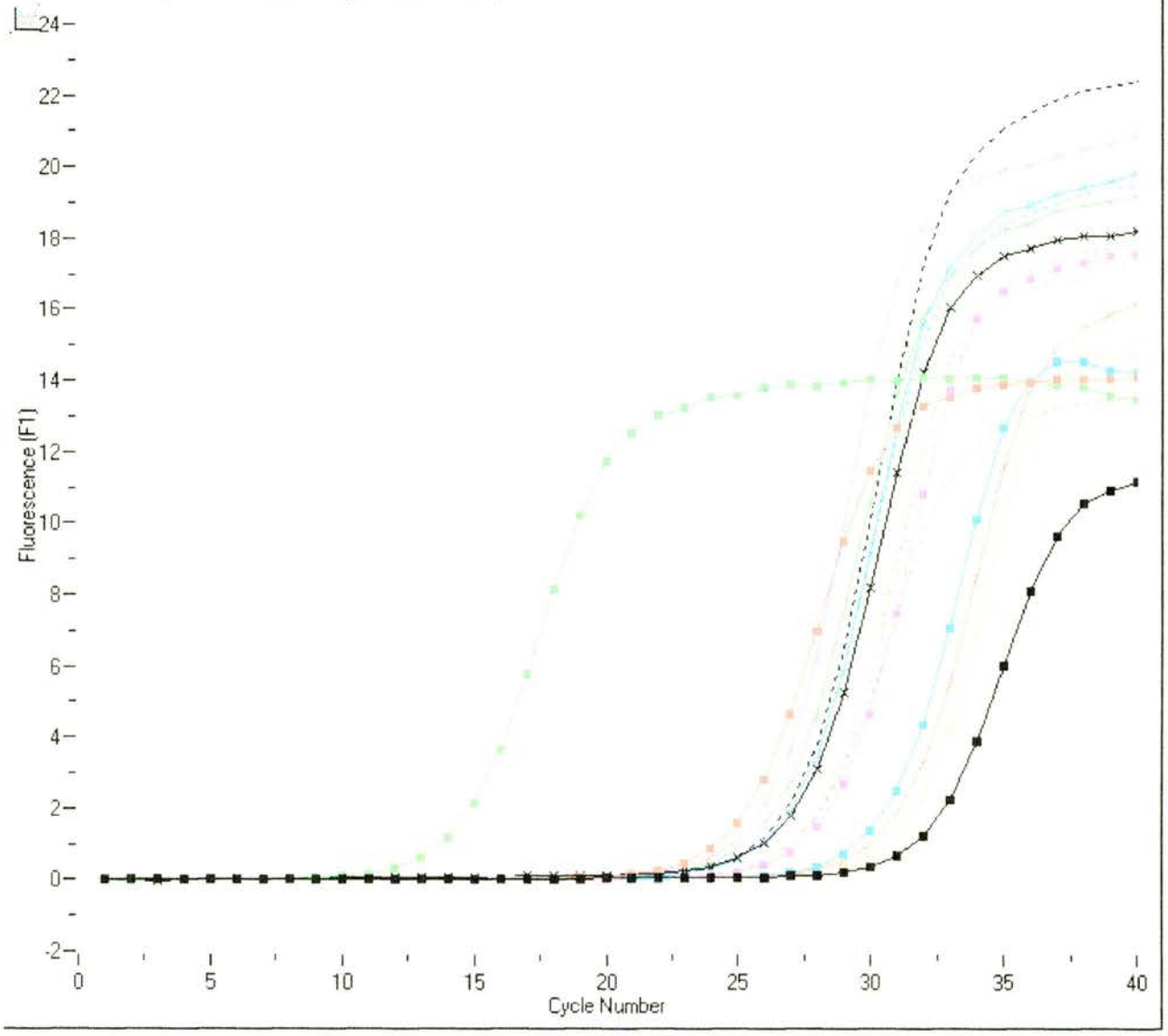
Number of Points: 2

☐ Show Fit Points

Analysis Notes: c-kit large intestine - PCR

P...	Name	Standard	Calculat...	Cro...
1	WISPA		3.556E+07	22.88
2	99/788		6.670E+07	23.82
3	03/488		6.980E+07	23.89
4	03/303		5.796E+07	23.61
5	03/381		2.491E+07	22.35
6	WOBBLER		1.412E+07	21.51
7	03/124		5.564E+07	23.55
8	03/242		2.416E+07	22.31
9	27644		2.893E+07	22.58
10	03/340		1.533E+07	21.63
11	18/5		4.555E+08	26.69
12	PEDRITA		1.256E+07	21.33
13	28570		1.132E+07	21.18
14	28535		8.328E+07	24.15
15	ANNIE B		7.701E+08	27.47
16	28225		1.751E+08	25.26
17	28287		3.677E+08	26.37
18	CKIT4	1.000E+04	9.749E+03	10.65
19	CKIT7	1.000E+07	1.107E+07	21.14
20	CKIT8	1.000E+08	9.265E+07	24.31
21	WATER		1.406E+09	28.37

Step 1: Baseline | Step 2: Noise Band | Step 3: Analysis



23

Start | Administrator | Front | LCDA | 14:50

File Quantification Report Window Help

nalysis

Fit Points

Second Derivative Maximum

Baseline Adjustment
- None
- Arithmetic
- Proportional
- Normalized

Number of Points: 2

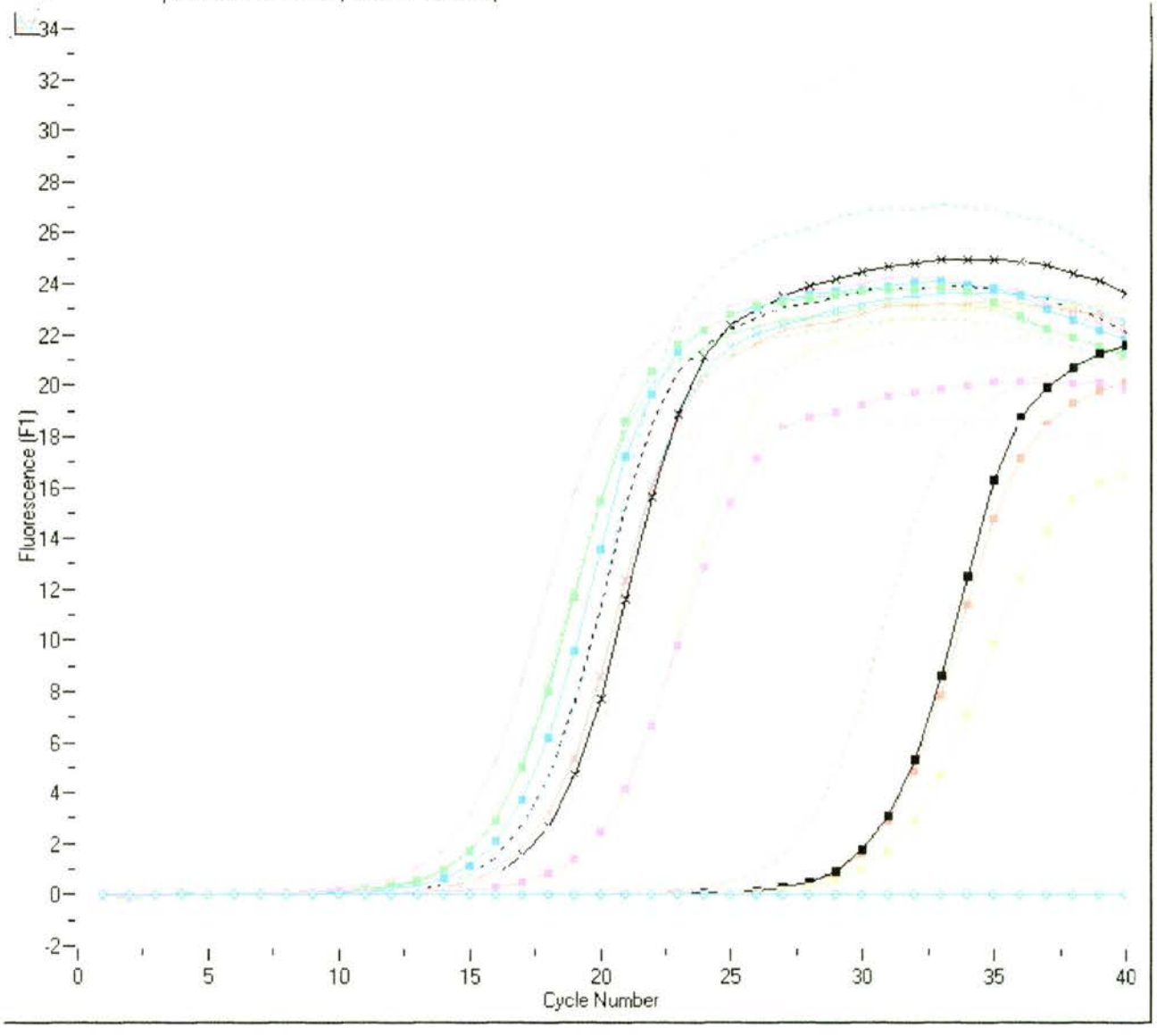
Show Fit Points

Analysis Notes: β-actin large intestine -PCR

P...	Name	Standard	Calculat...	Cro...
1	03/488il		2.202E-04	11.80
2	01/488il		5.952E-04	10.40
3	kbil		1.230E-07	22.33
4	02/56il		1.020E-04	12.88
5	wispapf		2.289E-04	11.75
6	99/788		1.042E-04	12.85
7	03/488		1.983E-04	11.95
8	03/303		4.335E-04	10.85
9	03/381		1.388E-04	12.45
10	wobbler		4.748E-04	10.72
11	03/124		1.286E-04	12.56
12	01/242		1.792E-03	8.853
13	27644		1.052E-04	12.84
14	03/340		2.088E-04	11.87
15	18/5		1.962E-05	15.20
16	pedrita		2.659E-04	11.53
17	28570		3.571E-04	11.12
18	28535		1.528E-03	9.078
19	annieb		1.337E-08	25.44
20	28225		5.000E-05	13.88
21	28287		1.191E-08	25.61
22	bact4	1.000E-04	1.051E-04	12.84
23	bact8	1.000E-08	7.790E-09	26.20
24	bact9	1.000E-09	1.221E-09	28.81
25	water			

222

Step 1: Baseline | Step 2: Noise Band | Step 3: Analysis



Start | Administrator | Front | LCDA | 14:56

LightCycler Data Analysis (Administrator) - [Melting Curves: CKITIL NC2; F1; MELTING CURVE seg 3]

File Melting Curves Report Window Help

Step 1: Melting Peaks | Step 2: Peak Areas | Extra: Manual Tm

Calculation Method
- Linear
- Linear with Background Correction
- Polynomial
- Polynomial with Background Correction

Digital Filter
- Enable

Analysis Notes

c-kit small intestine - melting curve

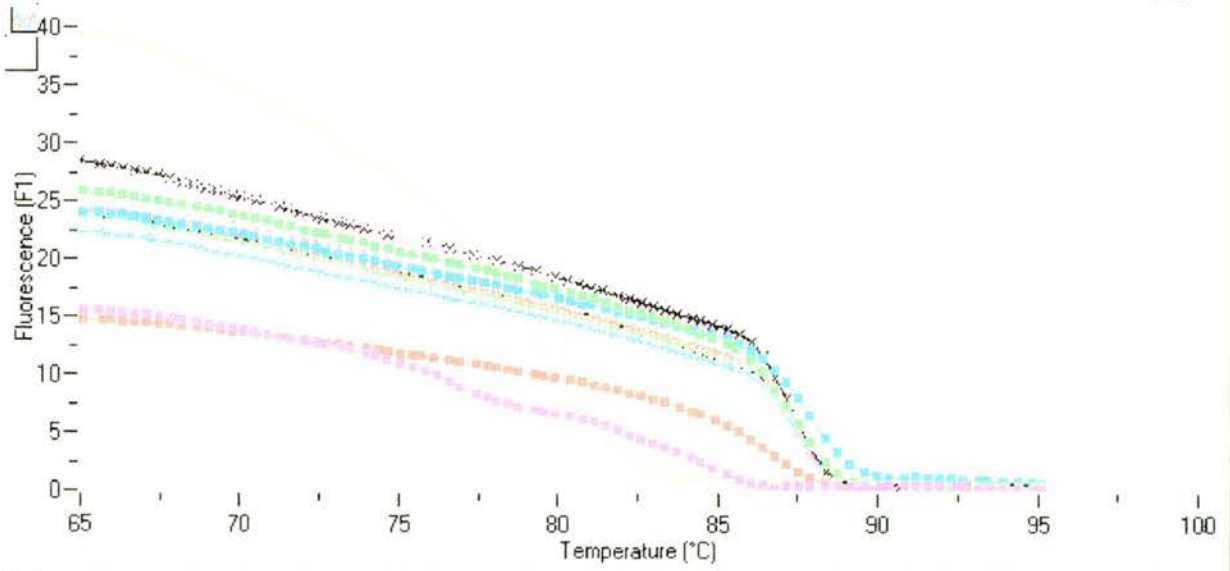
P...	Name	Standard
1	SSPCA	
2	03/488	
3	01/488	
4	KB	
5	02/56	
6	LAMB	
7	01/462	
10	03/381	
11	00/656	
12	02/90	
13	9/6/03	
14	03/435	
15	02/110	
16	02/97	
17	03/209	
18	27870	
19	C-KIT5	1.000E-05
20	C-KIT7	1.000E-07
21	C-KIT9	1.000E-09
22	WATER	

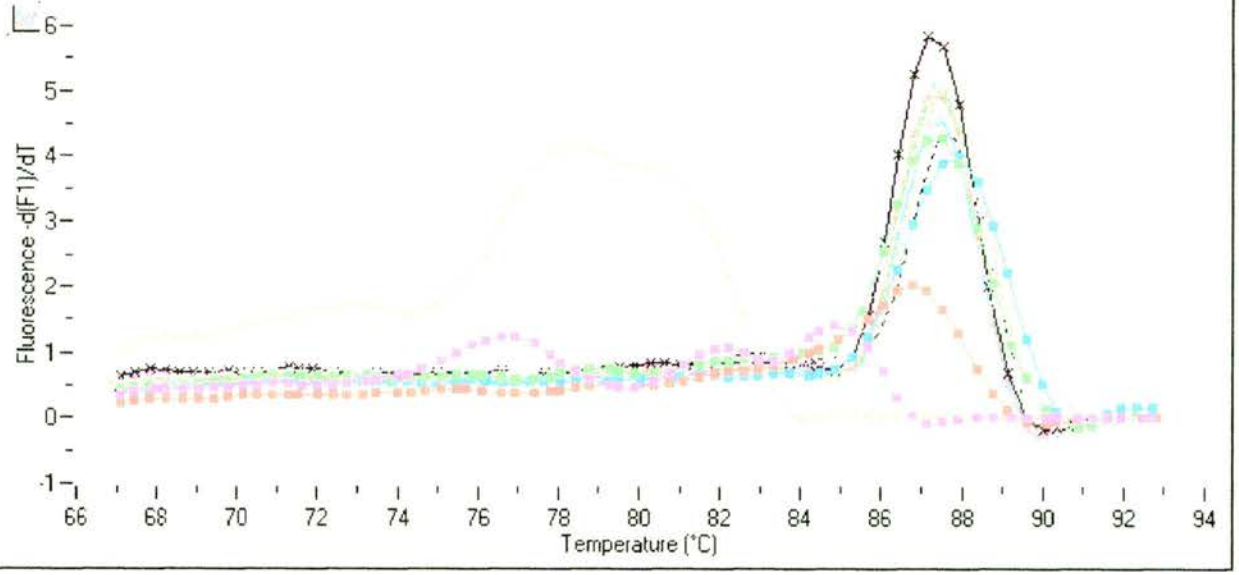
C° to Average

4.0

13.4

3.4





223

Start | Administrator | Data | Front | LCDA | 14:40

LightCycler Data Analysis (Administrator) - [Melting Curves: Bactinpiz; F1; MELTING CURVE Seg 3]

File Melting Curves Report Window Help

Step 1: Melting Peaks | Step 2: Peak Areas | Extra: Manual Tm

Calculation Method

- Linear
- Linear with Background Correction
- Polynomial
- Polynomial with Background Correction

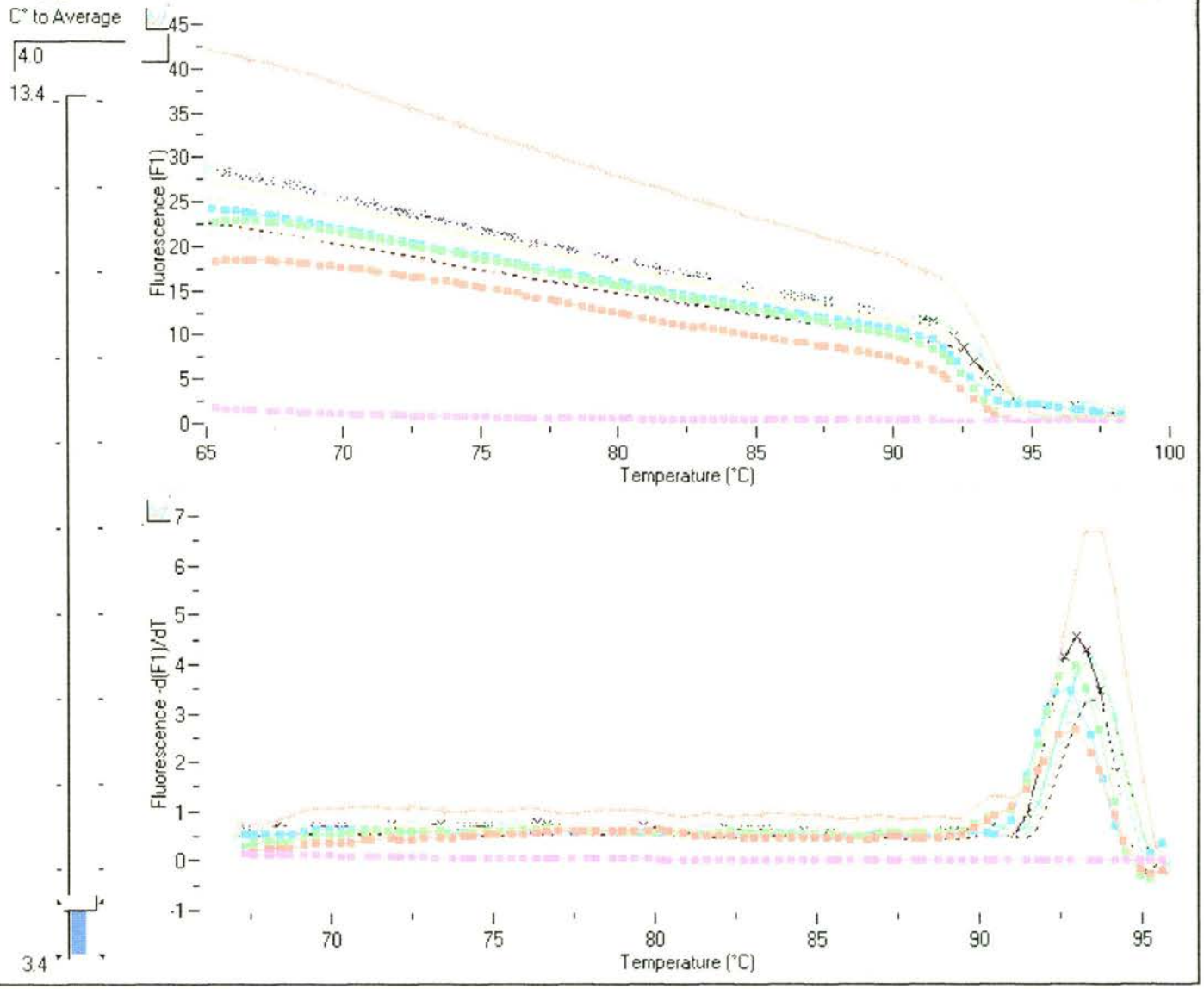
Digital Filter

☑ Enable

Analysis Notes

β-actin small intestine - melting curve

P...	Name	Standard
1	sspca old	
2	03/488	
3	01/488	
4	kb	
5	02/56	
6	lamb	
7	01/462	
8	03/381	
9	00/656	
10	02/90	
11	9/6/03	
12	03/435	
13	02/110	
14	02/97	
15	03/209	
16	27870	
17	bact4	1.000E-04
18	bact8	1.000E-08
19	bact9	1.000E-09
20	water	



228

Start | Administrator | Front | LCDA | 14:47

LightCycler Data Analysis (Administrator) - [Melting Curves: CKIT PFNL; F1; MELTING CURVE seg 3]

File Melting Curves Report Window Help

Step 1: Melting Peaks | Step 2: Peak Areas | Extra: Manual Tm

Calculation Method

- Linear
- Linear with Background Correction
- Polynomial
- Polynomial with Background Correction

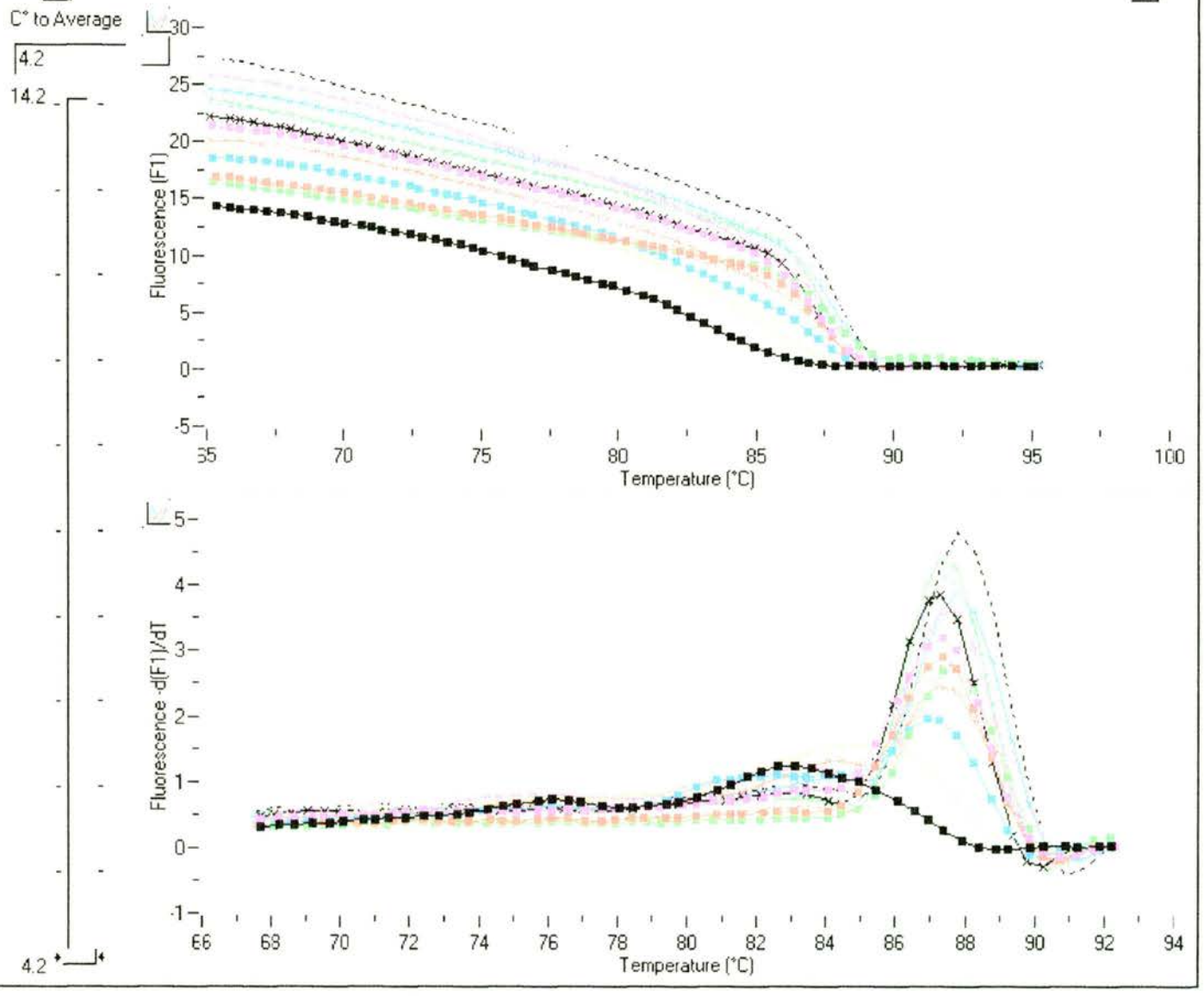
Digital Filter

☑ Enable

Analysis Notes

C-kit large intestine - melting curve

P...	Name	Standard
1	WISPA	
2	99/788	
3	03/488	
4	03/303	
5	03/381	
6	WOBBLER	
7	03/124	
8	03/242	
9	27644	
10	03/340	
11	18/5	
12	PEDRITA	
13	28570	
14	28535	
15	ANNIE B	
16	28225	
17	28287	
18	CKIT4	1.000E+04
19	CKIT7	1.000E+07
20	CKIT8	1.000E+08
21	WATER	



225

Start | Administrator | Front | LCDA | 14:52

LightCycler Data Analysis (Administrator) - [Melting Curves: bactiln pfnc; F1; MELTING CURVE seg 3]

File Melting Curves Report Window Help

tep 1: Melting Peaks | Step 2: Peak Areas | Extra: Manual Tm

Calculation Method

- Linear
- Linear with Background Correction
- Polynomial
- Polynomial with Background Correction

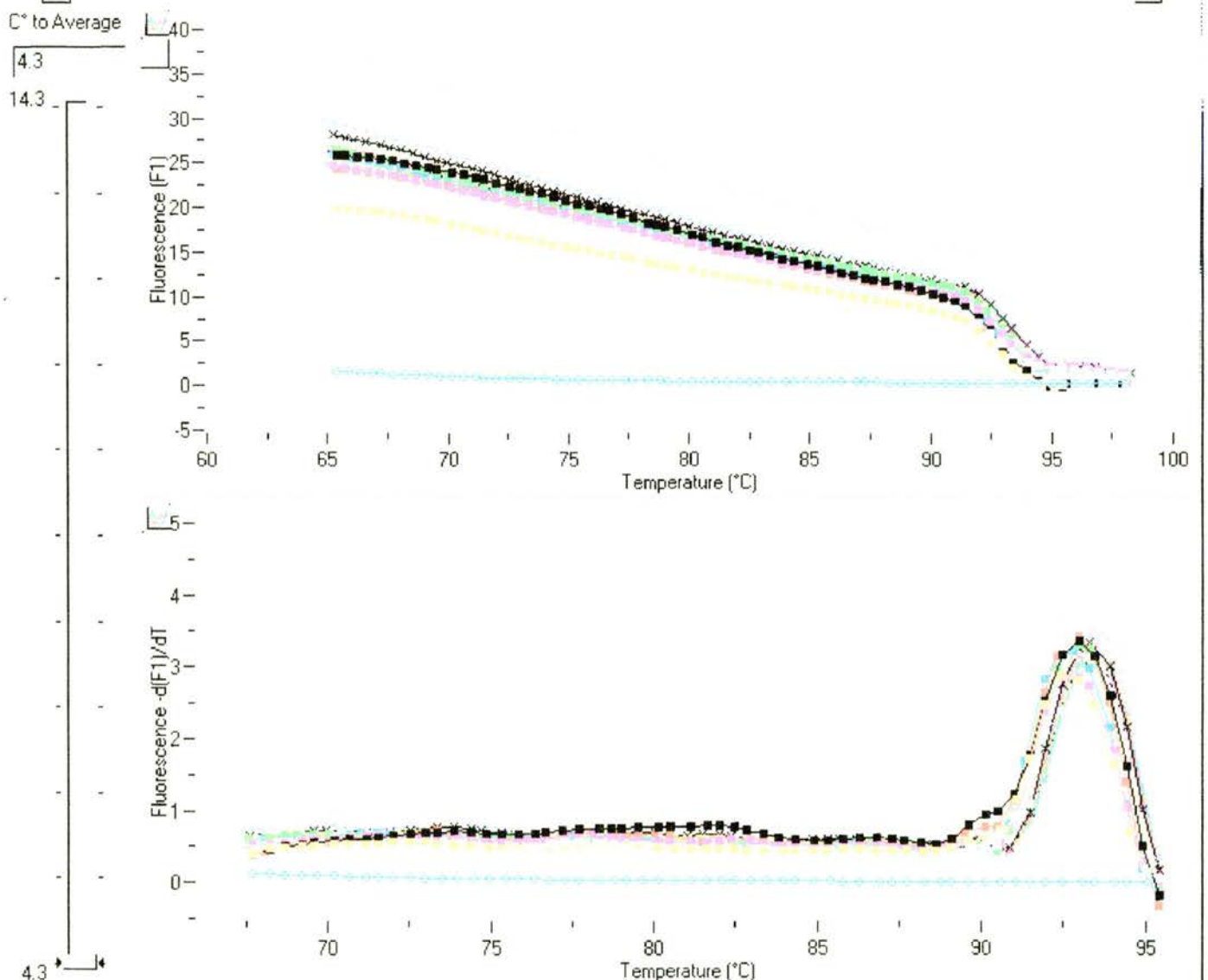
Digital Filter

☑ Enable

Analysis Notes

β-actin large intestine - melting curve

P...	Name	Standard
1	03/488il	
2	01/488il	
3	kbil	
4	02/56il	
5	wispapf	
6	99/788	
7	03/488	
8	03/303	
9	03/381	
10	wobbler	
11	03/124	
12	01/242	
13	27644	
14	03/340	
15	18/5	
16	pedrita	
17	28570	
18	28535	
19	annieb	
20	28225	
21	28287	
22	bact4	1.000E-04
23	bact8	1.000E-08
24	bact9	1.000E-09
25	water	



226

Start | Administrator | Front | LCDA | 15:01

APPENDIX 5:

Publications arising from work described in this thesis.

Fintl C, Pearson GTP, Mayhew IG, Ricketts SW, Hudson NPH (2004) The development and distribution of the interstitial cells of Cajal in the intestine of the equine foetus and neonate. *J. Anat.* **205**, 35-44.

Fintl C, Hudson NPH, Mayhew IG, Edwards GB, Proudman CJ, Pearson GTP (2004) The interstitial cells of Cajal (ICC) in equine colic: an immunohistochemical study of horses with obstructive disorders of the small and large intestines. *Equine vet. J.* **36**, 474-479.

Fintl C, Hudson NPH, Mayhew IG, Edwards GB, Proudman CJ, Pearson GTP (2003) The interstitial cells of Cajal (ICC) in equine colic: an immunohistochemical study of horses with obstructive disorders of the small and large intestines. Abstract BEVA Conference September 2003, Birmingham, pp283.

Fintl C, Pearson GTP, Mayhew IG, Ricketts SW, Hudson NPH (2004) Development of the interstitial cells of Cajal in the equine foetus: an immunohistochemical study. *J. Phys.* **555P**, 3P.

Hudson NPH, Fintl C, Mayhew IG, Pearson GTP (2003) The interstitial cells of Cajal and electrical activity of the equine intestine in health and disease. *Neurogastroent. Motil.* **15**, 225.

Milne EM, Fintl C, Mayhew IG, Hudson NPH, Pearson GT, Hahn CN (2004) Interstitial cells of Cajal, neurons and muscle– lessons from a recovered case of equine dysautonomia (grass sickness). *J. Comp. Path.* Submitted.

Fintl C, Lowden SL, Hopwood P, Palgrave CH, Hudson NPH, Mayhew IG, Edwards GB, Proudman CJ, Pearson GTP (2004) Comparative analysis of c-*kit* gene expression and c-Kit immunoreactivity in horses with and without intestinal disease. In preparation.

J. Anat. (2004) **205**, pp35–44

The development and distribution of the interstitial cells of Cajal in the intestine of the equine fetus and neonate

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Abstract

This study set out to determine the pattern of development and distribution of the interstitial cells of Cajal (ICC) in the intestinal tract of the equine fetus and neonate. Intestinal tissue samples from 12 naturally aborted equine fetuses and three euthanized neonates were collected and fixed in formalin prior to applying standard immunohistochemical labelling techniques targeting the c-Kit protein of the ICC. At 6 months of gestation, a network of ICC was present in the myenteric plexus region of both the small and the large intestine. ICC were also present within the circular muscle layer. In the large intestine, a proximal to distal gradient of distribution was evident, with few ICC observed in the more distal parts of the large intestine in the younger fetuses compared with the near-term animals. A transmural gradient of distribution was also evident within the large intestine, with the most luminal part of the muscularis externa being the last area to be colonized by ICC. This region did not appear fully developed until the early neonatal period. An increased density of ICC was noted throughout the large intestine in the regions of the taenial bands in all animals. This study is the first to describe ICC development and distribution in the equine fetus and neonate.

Key words c-Kit; foal; horse; immunohistochemistry; intestinal smooth muscle.

Introduction

The interstitial cells of Cajal (ICC) generate pacemaker activity throughout the gastrointestinal tract by initiating the slow wave activity that is integral to the coordination of gastrointestinal motility (Thuneberg, 1982; Hara et al. 1986; Ward et al. 1991; Sanders, 1996). In addition, ICC are thought to be involved in mediating neurotransmission and facilitating active propagation of electrical events (Thuneberg, 1982; Daniel & Posey-Daniel, 1984; Sanders et al. 1990; Daniel et al. 1998; Wang et al. 1999, 2000). It has been proposed that this wide range of functions is carried out by separate classes of ICC (Sanders, 1996). Recently, ICC have also been identified in the horse (Hudson et al. 1999).

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Accepted for publication *7 June 2004*

As no previous work has been undertaken to investigate the normal development of ICC in the horse, all current knowledge is based on comparative studies primarily in the mouse, rat and chicken (Torihashi et al. 1995, 1997; Lecoin et al. 1996; Young et al. 1996; Kluppel et al. 1998). There have also been a number of human clinical studies demonstrating that ICC, or precursors of ICC, start to colonize the intestinal tract at an early stage of fetal development (Kenny et al. 1998a,b, 1999; Wester et al. 1999). Generally, this seems to occur about one-third to half way through gestation, with some variation between species (Lecoin et al. 1996; Young et al. 1996; Torihashi et al. 1997; Kenny et al. 1999; Wester et al. 1999).

ICC express the proto-oncogene *c-kit*, which encodes c-Kit, a receptor tyrosine kinase (Ward et al. 1994; Huizinga et al. 1995; Torihashi et al. 1995). The c-Kit protein can be used as a marker for these cells using standard immunohistochemical labelling techniques (Sanders, 1996). Prior to the use of c-Kit immunohistochemistry, developmental studies of ICC were difficult to perform because definitive identification required

ultrastructural analysis (Faussone-Pellegrini, 1984, 1987; Faussone-Pellegrini et al. 1985). Furthermore, immature ICC possess few of the ultrastructural features associated with mature ICC, making ultrastructural identification difficult (Faussone-Pellegrini et al. 1996). The use of c-Kit as a marker for ICC not only enabled easier identification of the cells but also allowed earlier detection during their development (Lecoin et al. 1996; Young et al. 1996; Torihashi et al. 1997).

In support of a pacemaker-generating function of the ICC, it has been shown that the onset of electrical rhythmicity and slow wave activity occurs after the development of ICC networks, although there appears to be anatomical and interspecies variation regarding the exact timing of slow wave onset (Torihashi et al. 1997; Ward et al. 1997). An oral to anal temporal gradient in the functional development of ICC in the mouse has been reported in one study (Ward et al. 1997).

Other studies have demonstrated that concomitant with abnormal ICC morphology, numbers or function, there is a corresponding lack of normal electrical patterns in the intestinal tract, providing further evidence for the role of ICC as pacemaker cells (Ward et al. 1994; Huizinga et al. 1995; Torihashi et al. 1995). The abnormal development of ICC has been implicated in a number of motility disorders in the human neonate, including hypertrophic pyloric stenosis (Langer et al. 1995), anorectal malformations (Kenny et al. 1998a) and transient neonatal intestinal pseudo-obstruction (Kenny et al. 1998b; Yamataka et al. 1998).

There are several well-recognized gastrointestinal disturbances observed in the equine neonate, including meconium impaction (Palmer, 1985), intestinal malformations and intussusceptions (Greet, 1992). Other disturbances include abnormal motility patterns and ileus typically observed in premature and dysmature foals (Wilson & Cudd, 1990) as well as in foals with hypoxic ischaemic insults in the immediate peripartum period (Vaala, 1994).

The purpose of this study was to determine the pattern of development and distribution of ICC in the normal equine fetus and neonate.

Materials and methods

Animals, sampling and processing

Surplus intestinal tissue samples from 22 naturally aborted equine fetuses were collected during routine abortion investigations. Two fetuses were examined as part of a post-mortem investigation associated with the death of the mare. The ages of the fetuses ranged from 6 to 11 months of gestation. A wide range in gestational length has been described in Thoroughbreds (320–360 days, mean 340 days) and ponies (315–350 days, mean 333 days) (Rossdale et al. 1991; Rossdale, 1993). Two fetuses included in this study were aborted from pony mares, and the remaining animals were all Thoroughbreds. As there was sometimes a delay before the fetus was presented for post-mortem examination, ten of the 22 fetuses had to be excluded from the study owing to the degree of tissue autolysis (see below).

In addition, surplus intestinal tissue samples were collected from three euthanized foals aged 4, 7 and 8 weeks postpartum, following the routine post-mortem investigations of these animals. One foal had contracted Tyzzer's disease (a bacterial hepatitis), one had an intestinal volvulus of the proximal small intestine (at a site distant to the collected tissue), and the third foal was euthanized due to severe orthopaedic disease.

Samples of intestine were collected from all animals at anatomically defined sites. From the small intestine, this was ileum (at a position level with the midpoint of the ileocaecal fold); from the large intestine, this included caecal base, pelvic flexure apex (located at the junction between the left ventral colon and left dorsal colon), and the distal third of the small colon (equivalent to the descending colon in humans).

Following collection, all samples were immediately placed in 10% phosphate-buffered formalin and fixed for at least 24 h. Tissue processing consisted of rinsing the tissue samples in running tap water for 1 h prior to soaking in graded sucrose solutions (10 and 30% sucrose in phosphate-buffered saline, PBS), in order to cryoprotect the samples. The samples were frozen rapidly in isopentane precooled in liquid nitrogen and subsequently cut at thicknesses of 10 μm and 20 μm. All sections were mounted on Tespa-coated (3-aminopropyltriethoxysilane) (Sigma, Poole, UK) slides and allowed to air-dry overnight. After washing the sections in PBS, sections were incubated for 30 min in 0.3% hydrogen peroxide in methanol to quench endogenous peroxidase activity. Sections were then incubated for 1 h in 1% goat serum (Vector Laboratories, Burlingame, CA, USA) to block non-specific antibody binding. After subsequent washes with PBS, the sections were incubated overnight at 4 °C in humid chambers in a rabbit-raised polyclonal antiserum to c-Kit (Ab-1) (Oncogene Research

Products, Cambridge, MA, USA) at a concentration of 1 μg mL^{-1}. Further washing in PBS was followed by a 1-h incubation with biotin-conjugated goat anti-rabbit immunoglobulin (Vector Laboratories) at a dilution of 1 : 200. Immunoreactivity was revealed using the avidin–biotin method (ABC) (Vectastain Elite ABC Kit, Vector Laboratories) using a diaminobenzidine substrate (DAB) (BDH Laboratory Supplies, Poole, UK). Sections were dehydrated in ethanol, cleared in xylene and then mounted in Depex (Merck, Glasgow, UK).

For all batches processed, both negative and positive controls were included. In negative controls, the primary antibody was replaced with normal rabbit serum and resulted in complete absence of immunolabelling. Tissue samples from a normal control animal from a previous study that had been shown to have an abundance of c-Kit immunoreactivity were used as positive controls.

ICC were differentiated from round c-Kit-immunoreactive mast cells in the submucosa by their morphology (e.g. presence of cellular processes in ICC), or by staining parallel sections with toluidine blue to demonstrate mast cell metachromasia (Galli et al. 1993; Hudson et al. 1999).

In addition to immunohistochemical labelling, all processed tissues were stained with haematoxylin and eosin in order to assess tissue integrity. This was carried out to help clarify whether reduced or absent c-Kit immunoreactivity was a primary finding or secondary to a degree of tissue autolysis. Only samples with good tissue integrity (and not worse than a mild degree of mucosal degradation) were included in order to ensure that immunohistochemical grading was reliable. Ten fetuses were rejected from the study on this basis. For analysis, the 12 fetuses and three foals were grouped together in different age groups as described in Table 1.

Results

In the small intestine (ileum) of all the fetuses examined, c-Kit-immunoreactive ICC were identified in the myenteric plexus region displaying a mixture of bipolar- and stellate-shaped morphologies. Individual ICC could be identified in the youngest fetuses but, in contrast, in the older fetuses and foals, the increasingly dense ICC network that developed surrounding the myenteric ganglia made it more difficult to identify individual cells (Figs 1 and 2). There was a limited and inconsistent degree of branching of ICC from the myenteric plexus region into the longitudinal muscle layer in the fetuses. However, this branching became more prominent and consistent in the neonates (Fig. 2).

Interstitial cells were also observed in the small intestinal circular muscular layer (Figs 1 and 2) in all samples. In the youngest fetus (6 months of gestation), few cells were observed within this muscle layer compared with the older fetus and neonate (Figs 1 and 2). The majority of ICC in the circular muscle of the ileum were bipolar in shape, although in the older fetus there appeared to be a slight increase in the proportion of stellate-shaped cells. The orientation of the long axis of ICC was parallel to the circular muscle fibres.

In the large intestine (caecum, pelvic flexure and small colon), both bipolar- and stellate-shaped c-Kit-immunoreactive ICC were present in the myenteric plexus region, with no predominance of either morphology. The ICC did not form the dense networks observed in the small intestine. The distribution and density of ICC in this region did not appear to change significantly during fetal development or indeed into the neonatal period (see Fig. 6).

Bipolar- and stellate-shaped ICC were also observed in the circular muscle layer of the muscularis externa of the large intestine in all samples (Fig. 3). However, in contrast to the ileum, there was no clear predominance of either morphological type. A proximal to distal gradient of ICC distribution was detected, with more ICC present in the proximal large intestine than in the distal large intestine. This gradient was most evident in the youngest fetuses (6–8 months of gestation) but became less distinct as the fetuses developed (Fig. 4). A summary of this proximal to distal gradient of distribution is illustrated later in Fig. 6.

Table 1 Age groups of fetuses and foals

Fetal/foal age	F6–F7	F8–F9	F10–F11	Full term	4–8 weeks postpartum
No. of animals	2	4	4	2	3

Key: F6–7, 6th and 7th month of fetal development, etc.

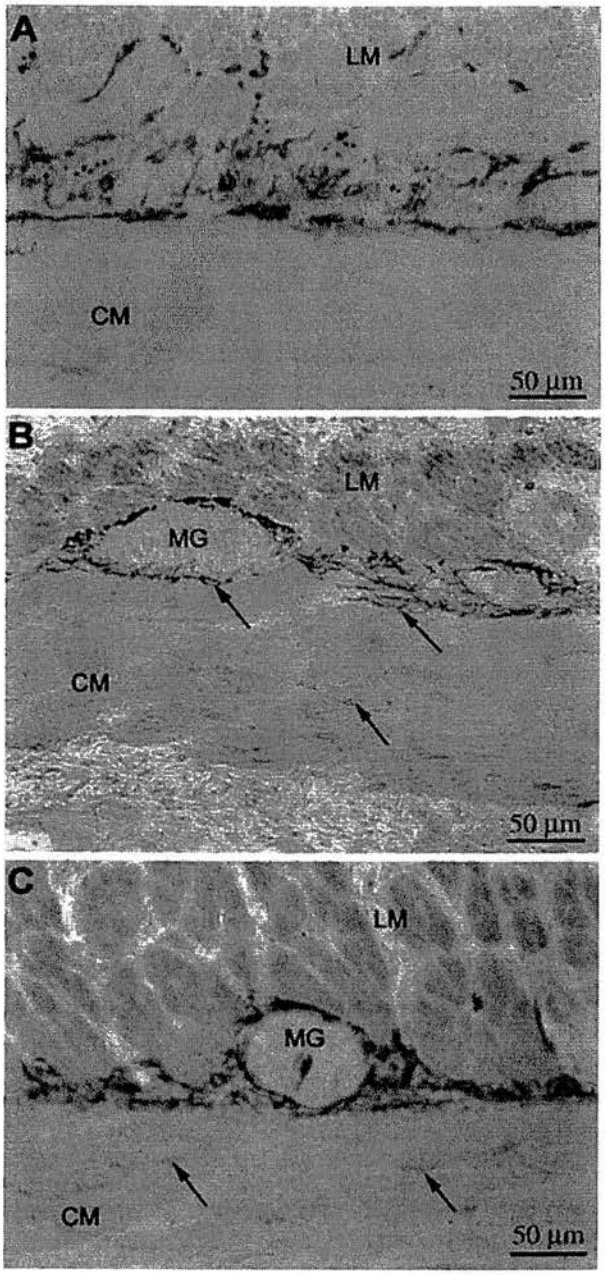


Fig. 1 c-Kit immunoreactivity in the ileum of the small intestine in three fetuses, at 6 months (A), 7 months (B) and 10 months (C) of gestation. Individual c-Kit-immunoreactive ICC can be observed in both the myenteric plexus region and the circular muscle layer (arrows). LM, longitudinal muscle layer; CM, circular muscle layer; MG, myenteric ganglia.

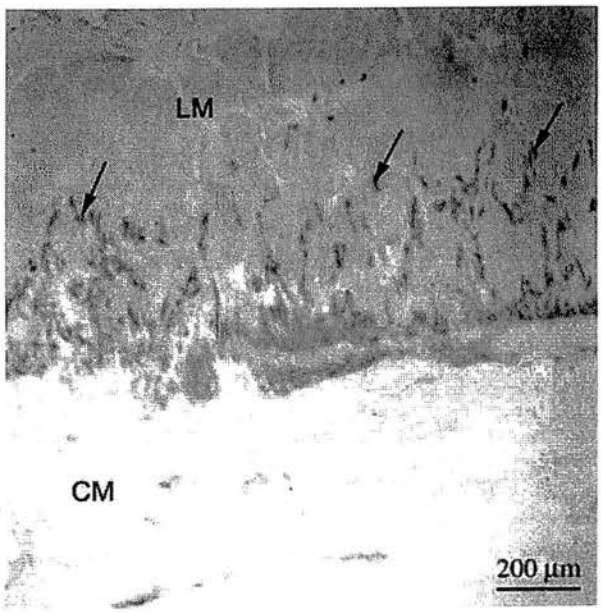


Fig. 2 c-Kit immunoreactivity in the ileum of the small intestine in a 2-month-old foal. A dense network of ICC surrounds the myenteric ganglia with branching of ICC from the myenteric plexus region into the longitudinal muscle layer (arrows). LM, longitudinal muscle layer; CM, circular muscle layer.

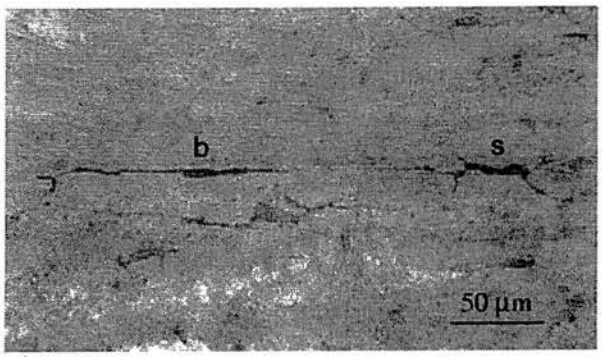


Fig. 3 Bipolar-shaped (b) and stellate-shaped (s) c-Kit-immunoreactive ICC in the large intestine of a fetus at 6 months of gestation. The ICC shown are located in the circular smooth muscle layer of the pelvic flexure and are typical of those seen elsewhere in the small and large intestines of the horse.

In addition, a transmural gradient of ICC distribution was present in the circular muscle layer of the large intestine. In the youngest fetuses, the majority of ICC in the most proximal part of the large intestine (caecum) were seen in the outer (more serosal) two-thirds of this muscle layer, with the innermost region having a lower ICC density (Fig. 4A). However, at the same stage of gestation, in the most caudal part of the large intestine, the distal small colon, very few ICC were observed in any part of the circular muscle layer (Fig. 4B). In the developing fetus, the transmural distribution observed in the pelvic flexure (a site approximately midway

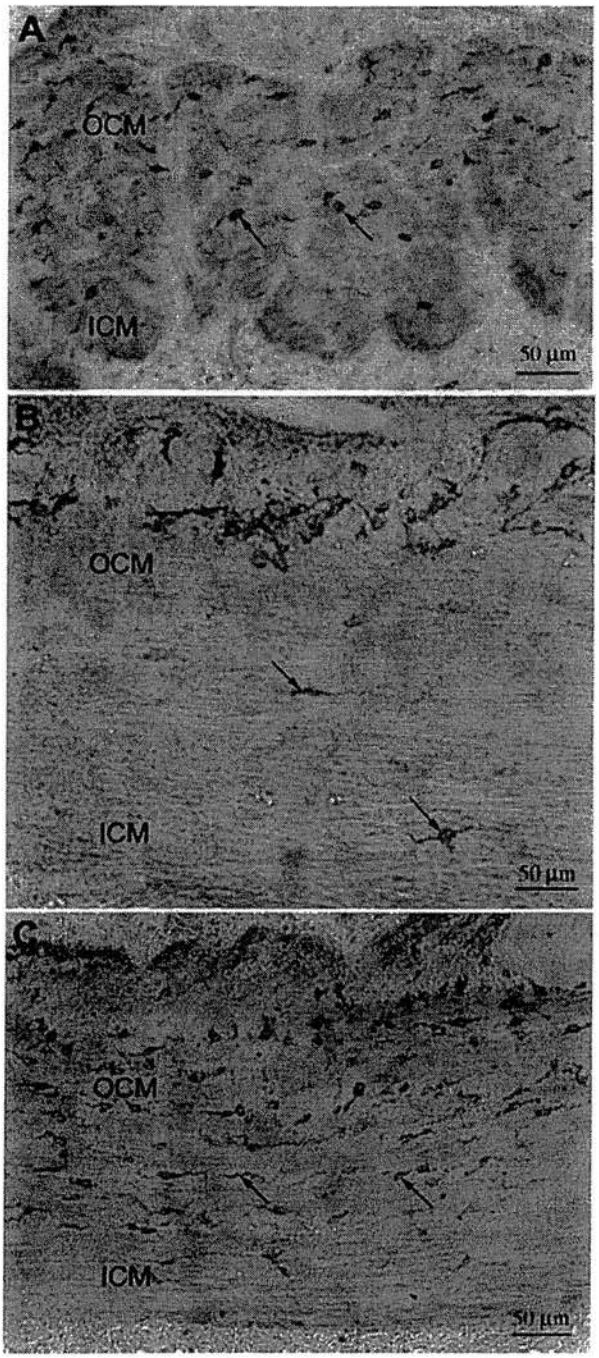


Fig. 4 c-Kit immunoreactivity in the large intestine of the fetus. (A) Caecal base at 7 months of gestation; (B) distal small colon at 7 months of gestation (same fetus as shown in A); (C) distal small colon at 10 months of gestation. OCM, outer region of circular muscle layer; ICM, inner region of circular muscle layer.

between the caecum and distal small colon) was more limited than in the caecum but more extensive than in the distal small colon. In late gestation, an area of low ICC density at the innermost part of the circular muscle

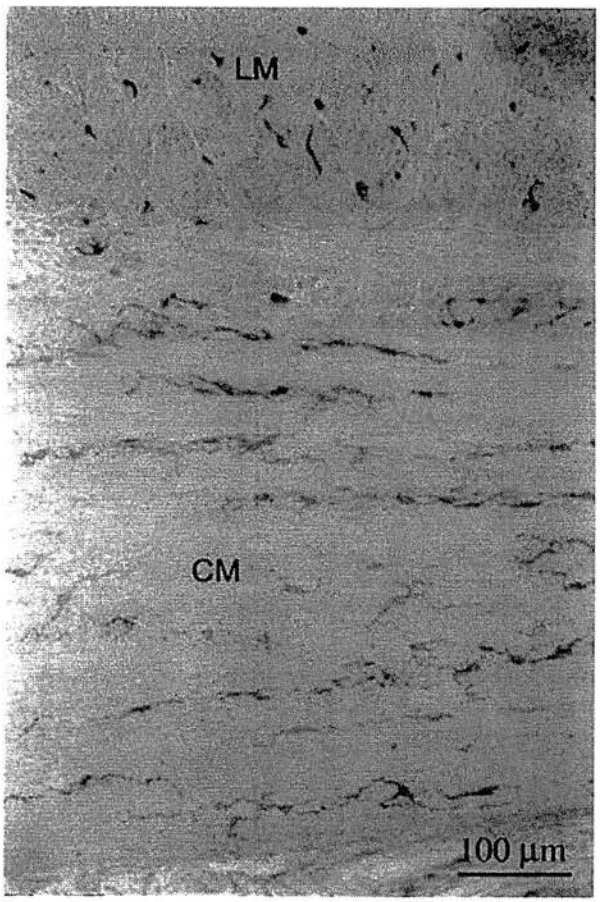


Fig. 5 c-Kit immunoreactivity in the distal small colon of a 2-month-old foal. The transmural gradient of ICC distribution seen in the developing fetus is no longer apparent and there is now a uniform distribution of ICC throughout the circular muscle layer. LM, longitudinal muscle layer; CM, circular muscle layer.

layer was still evident in the most caudal part of the large intestine (Fig. 4C). The transmural gradient of ICC distribution was no longer apparent in the neonatal animals (Fig. 5). A schematic representation of the transmural gradient is shown in Fig. 6.

In the areas where taenial bands were located, ICC density was consistently higher, both in the myenteric plexus region and in the circular muscle layer than in areas away from the taenial band (Fig. 7). This was found to be the case in both the developing fetus and the foals, irrespective of age and anatomical location.

Discussion

This is the first study to describe ICC development and distribution in the equine fetus and neonate.

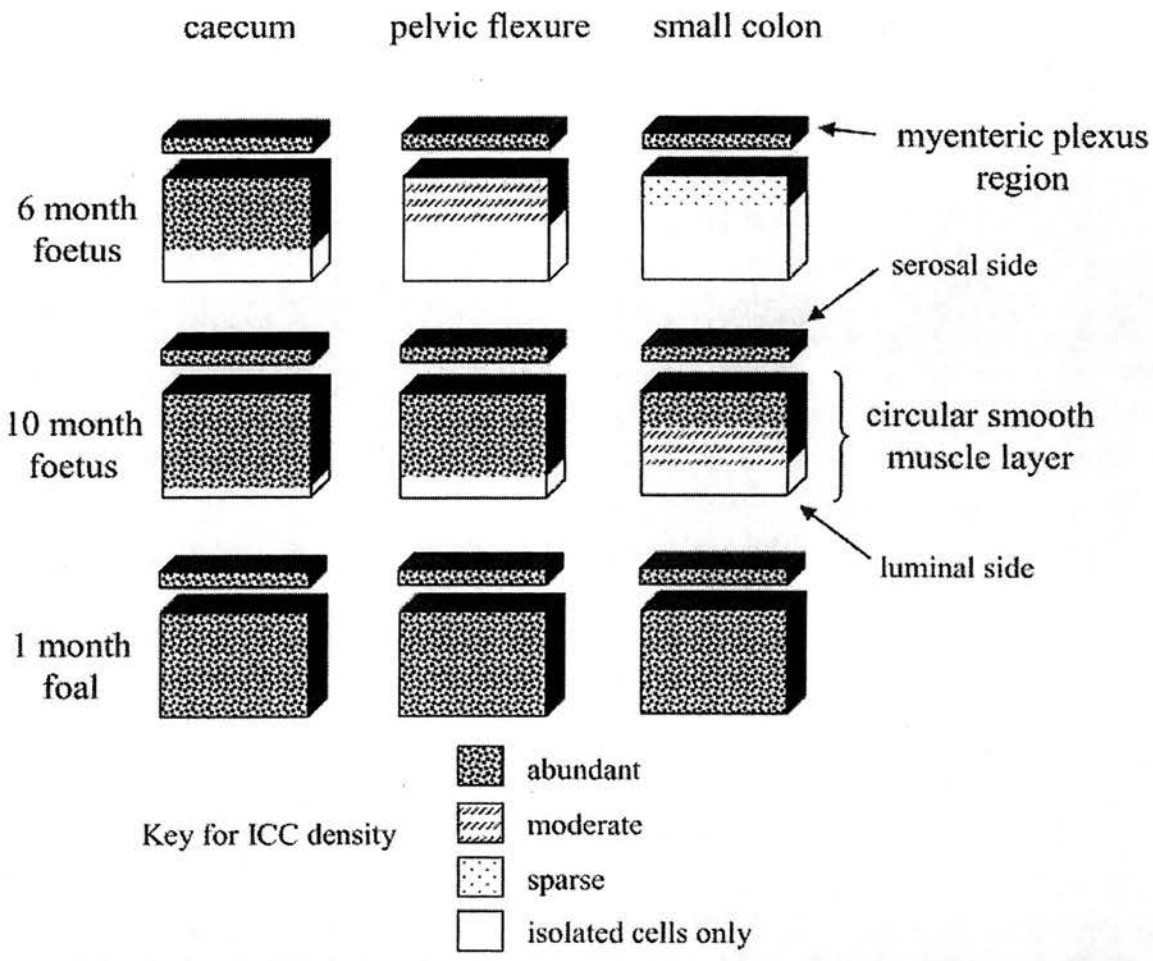
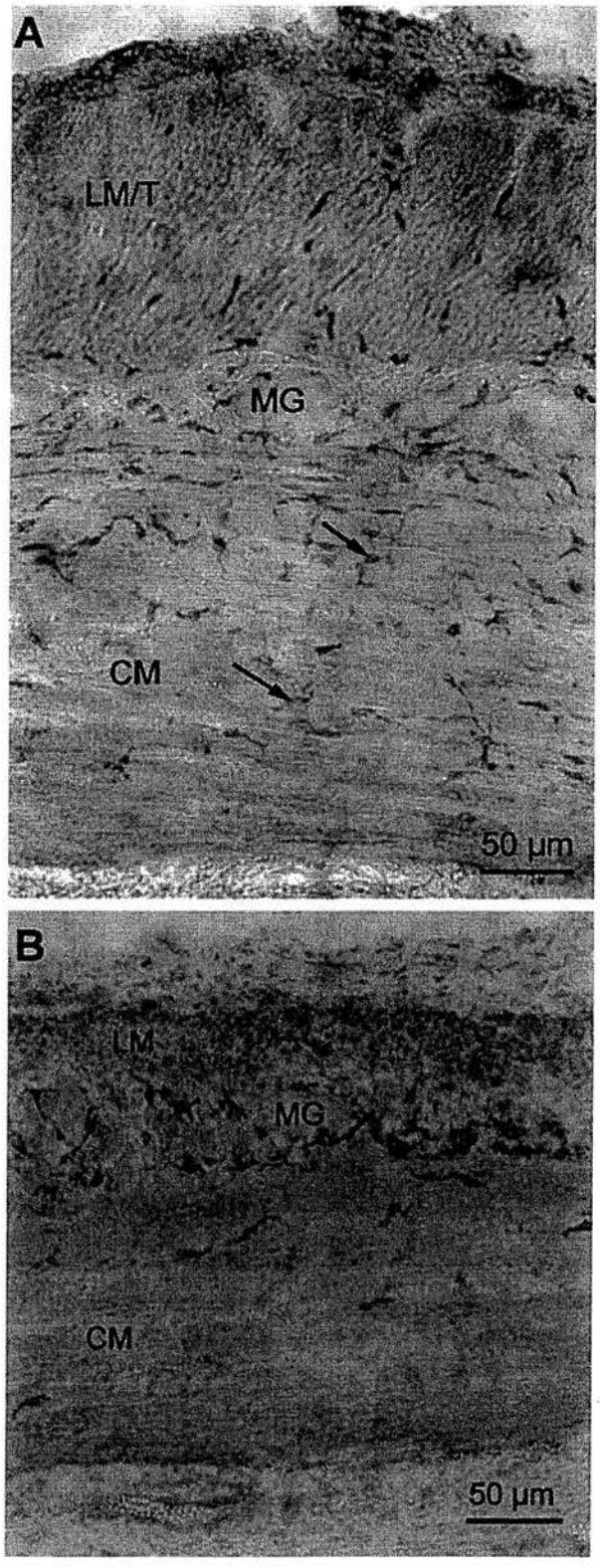


Fig. 6 Schematic illustration of the proximal to distal developmental distributions of ICC in the myenteric plexus region and the circular muscle layer of the equine large intestine. The age-related changes in the transmural distribution of ICC within the circular muscle layer are also illustrated.

Developmental studies in other species have demonstrated that ICC start to colonize the intestinal tract about one-third to half way through gestation (Lecoin et al. 1996; Young et al. 1996; Torihashi et al. 1997; Kenny et al. 1999; Wester et al. 1999) and it seems reasonable to speculate that a similar time-scale of development also occurs in the equine fetus. This would suggest that the initial colonization of the equine intestinal tract would commence around the 4th to 6th month of gestation. However, considering the wide range of normal gestational lengths in this species (Rossdale et al. 1991; Rossdale, 1993), there may also be some individual variation in the time of initial ICC colonization. Unfortunately, equine fetuses under 6 months of gestation were not available for this study, principally because many abortions at this stage of pregnancy occur undetected.

At full term, ICC distribution and density in the small intestine appeared to be well developed. The cells were detectable not only in dense networks in the myenteric plexus region but also in the circular muscle layer of the muscularis externa. This distribution differs somewhat from other species in which ICC in the circular muscle layer were only sparsely, if at all, detectable until the neonatal period (Faussone-Pellegrini, 1984; Torihashi et al. 1995, 1997; Young et al. 1996; Ward et al. 1997; Kenny et al. 1999; Wester et al. 1999).

It is thought that ICC located around the myenteric plexus in the small intestine are involved in generating pacemaker activity in this region, whereas those located in the circular muscle layer primarily mediate neurotransmission (Sanders, 1996). Assuming this also applies



to the horse, the findings in the current study suggest that, in the equine fetus, development of ICC classes of both the pacemaker-generating as well as the neurotransmission-mediating functions may be relatively advanced at the time of birth compared with other mammalian species. This apparent maturity is further supported by the observation that there did not appear to be significant changes in ICC density and distribution in the small intestine in the equine neonate compared with that of the full-term fetus. However, it is recognized that classification of the different types of ICC in the horse needs further investigation. For example, to investigate possible differences in function of the two morphological categories of ICC, it may be necessary to conduct both electrophysiological and electron microscopical studies (Rumessen et al. 1993; Liu et al. 1998).

The maturity of small intestinal ICC networks at full term differed somewhat from that of the large intestine. In the latter, a proximal to distal gradient of ICC distribution was evident, with the most distal part of the large intestine showing some evidence of ongoing development into the neonatal period. This type of developmental gradient has also been recognized in previous studies in other species (Faussone-Pellegrini et al. 1996; Ward et al. 1997; Kenny et al. 1998a). Ward et al. (1997) demonstrated that this developmental pattern also mirrored the onset of electrical rhythmicity in the murine gastrointestinal tract, providing further evidence that some of these cells generate pacemaker activity.

At full term, in the most distal part of the large intestine, there was incomplete ICC colonization of the innermost aspect of the circular muscle layer. Faussone-Pellegrini (1987) demonstrated in an ultrastructural study that the innermost aspect of the circular muscle layer in the mouse colon was the least differentiated layer at the time of birth when fibroblast-like cells still differentiated into smooth muscle cells. Lecoin et al. (1996) subsequently demonstrated that ICC were mesenchymal in origin and hence share a common developmental origin with smooth muscle cells and

Fig. 7 c-Kit-immunoreactivity in the distal small colon of a fetus at 7 months of gestation. (A) Taenial band region. Numerous ICC can be observed in the circular muscle layer (arrows) (B) away from the taenial band in the same animal. Note the lower ICC density in this region compared with A. LM/T, longitudinal muscle layer/taenial band; LM, longitudinal muscle; CM, circular muscle layer; MG, myenteric ganglia.

fibroblasts. Indeed, this close relationship was further demonstrated when Torihashi et al. (1999) noted that blockade of c-Kit signalling induced transdifferentiation of ICC into a smooth muscle phenotype, indicating that this signalling was important not only for differentiation but also for maintenance of the ICC phenotype. Considering this information, it is possible that ongoing differentiation through c-Kit development and signalling is taking place in this last part of the large intestine and is not complete until some time after birth. In the three neonatal animals examined, there appeared to be a uniform distribution of ICC throughout the circular muscle layer in the most distal part of the large intestine, indicating that this differentiation may be complete at this stage. The apparent immaturity at full term of the ICC in this region coupled with the proposed location of the main pacemaker-generating ICC at the submucosal border of the circular muscle layer in the large colon (Durdle et al. 1983; Ward & Sanders, 1990) offers possible insight into the causes of equine neonatal intestinal obstructive disorders such as meconium impaction. Assuming this area is also the primary pacemaker-generating region in the horse, this immaturity of ICC at birth may contribute to retention of meconium in the distal colon and rectum by some neonatal foals. However, it is possible that even immature cells may have functional properties as demonstrated by Torihashi et al. (1997) and hence functional electrophysiological studies are needed in order to determine the onset of normal motility patterns, as well to confirm the location of primary pacemaker sites in the distal large intestine of the foal.

In the colon of some species, including the dog (Berezin et al. 1988) and human (Rumessen, 1996), ICC have been described in association with the nerve fibres of the submuscular plexus on the luminal side of the circular muscle layer. Although no accumulation of ICC in this region has been seen in the current study and a submuscular plexus in the equine colon has yet to be identified, this is an area that warrants further investigation.

In the large intestine, there appeared to be an increased density of ICC in the circular muscle layer as well as in the myenteric plexus region in the areas near the taenial bands irrespective of gestational age or anatomical region. Increased ICC density in these areas has been described previously in adult horses (Hudson et al. 1999). The taenial bands are important as they are likely to be actively involved in peristalsis (Burns, 1992). A second pacemaker-generating area in the myenteric plexus region of the colon has been demonstrated in the dog, initiating myenteric potential oscillations that spread into both the circular and the longitudinal muscle layers (Smith et al. 1987). It is possible that ICC surrounding the myenteric plexus, especially in the region of the taenial bands, represent additional pacemaker-generating areas in the equine colon in addition to those proposed to be located in the circular muscle layer. This may account for the increased ICC density in these areas. A more likely explanation is that the ICC here are involved in mediating neurotransmission (Thuneberg, 1982; Daniel & Posey-Daniel, 1984). The high ICC density in the region of the taenial bands may therefore be related to the dense innervation that has been noted in this area (Burns, 1992).

In summary, this study has demonstrated the presence and distribution of ICC in the equine fetus during the latter half of gestation and in the neonate. It has shown that ICC distribution and density in the small intestine in the full-term fetus appear similar to that of the neonatal animal. However, it appears that ICC development in the distal large intestine continues after birth with further colonization of the inner aspect of the circular muscle region. This study has provided information on ICC development and distribution in the equine fetus and neonate that will provide a foundation for future studies in diseased animals.

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References

Berezin I, Huizinga JD, Daniel EE (1988) Interstitial cells of Cajal in the canine colon: a special communication network at the inner border of the circular muscle. *J. Comp. Neurol.* **273**, 42–51.

Burns GA (1992) The teniae of the equine intestine. *Cornell Vet.* **82**, 187–212.

Daniel EE, Posey-Daniel V (1984) Neuromuscular structures in opossum esophagus: role of interstitial cells of Cajal. *Am. J. Phys.* **246**, G305–G315.

Daniel EE, Wang Y-F, Cayabyab FS (1998) Role of gap junctions in structural arrangements of interstitial cells of Cajal and canine ileal smooth muscle. *Am. J. Phys.* **274**, G1125–G1141.

Durdle NG, Kingma YJ, Bowes KL, Chambers MM (1983) Origin of slow waves in the canine colon. *Gastroenterology* **84**, 375–382.

Faussone-Pellegrini MS (1984) Morphogenesis of the special circular muscle layer and of the interstitial cells of Cajal related to the plexus muscularis profondus of mouse intestinal muscle coat: an EM study. *Anat. Embryol.* **169**, 151–158.

Faussone-Pellegrini MS, Matini P, Stach W (1985) Differentiation of enteric plexuses and interstitial cells of Cajal in the rat gut during pre- and postnatal life. *Acta Anat.* **155**, 113–125.

Faussone-Pellegrini MS (1987) Cytodifferentiation of the interstitial cells of Cajal of mouse colonic circular muscle layer. *Acta Anat.* **128**, 98–109.

Faussone-Pellegrini MS, Matini P, Stach W (1996) Differentiation of enteric plexuses and interstitial cells of Cajal in the rat gut during pre- and postnatal life. *Acta Anat.* **155**, 113–125.

Galli SJ, Tsai M, Wershil BK (1993) The *c-kit* receptor, stem cell factor, and mast cells. *Am. J. Pathol.* **142**, 965–974.

Greet TR (1992) Ileal intussusception in 16 young thoroughbreds. *Equine Vet. J.* **24**, 81–83.

Hara Y, Kubota M, Szurszewski JH (1986) Electrophysiology of smooth muscle of the small intestine of some mammals. *J. Physiol.* **372**, 501–520.

Hudson NPH, Pearson GT, Kitamura N, Mayhew IG (1999) An immunohistochemical study of interstitial cells of Cajal in the equine gastrointestinal tract. *Res. Vet. Sci.* **66**, 265–271.

Huizinga JD, Thuneberg L, Kluppel M, Malusz J, Mikkelsen HB, Bernstein A (1995) W/kit gene required for interstitial cells of Cajal and for intestinal pacemaker activity. *Nature* **373**, 347–349.

Kenny SE, Connell MG, Rintala RJ, Vaillant C, Edgar DH, Lloyd DA (1998a) Abnormal colonic interstitial cells of Cajal in children with anorectal malformations. *J. Ped. Surg.* **33**, 130–132.

Kenny SE, Vanderwinden JM, Rintala RJ, Connell MG, Lloyd DA, Vanderhaegen JJ, De Laet MH (1998b) Delayed maturation of the interstitial cells of Cajal: a new diagnosis for transient neonatal pseudoobstruction. Report of two cases. *J. Ped. Surg.* **33**, 94–98.

Kenny SE, Connell G, Woodward MN, Lloyd DA, Gosden CM, Edgar DH, Vaillant C (1999) Ontogeny of the interstitial cells of Cajal in the human intestine. *J. Ped. Surg.* **34**, 1241–1247.

Kluppel M, Huizinga JD, Malysz J, Bernstein A (1998) Developmental origin and *kit*-dependent development of the interstitial cells of Cajal in the mammalian small intestine. *Dev. Dyn.* **211**, 60–71.

Langer JC, Berezin I, Daniel EE (1995) Hypertrophic pyloric stenosis: ultrastructural abnormalities of enteric nerves and the interstitial cells of Cajal. *J. Ped. Surg.* **30**, 1535–1543.

Lecoin L, Gabella G, LeDouarin N (1996) Origin of the *c-kit*-positive interstitial cells in the avian bowel. *Development* **122**, 725–733.

Liu LWC, Thuneberg L, Huizinga JD (1998) Development of pacemaker activity and interstitial cells of Cajal in the neonatal mouse small intestine. *Dev. Dyn.* **213**, 271–282.

Palmer JE (1985) Gastrointestinal diseases of foals. *Vet. Clin. North Am.* **5**, 151–168.

Rossdale PD, Ousey JC, Cottrill CM, Chavatte P, Allen WR, McGladdery AJ (1991) Effects of placental pathology on maternal plasma progestagen and mammary secretion calcium concentrations and on neonatal adrenocortical function in the horse. *J. Reprod. Fertil. Suppl.* **44**, 579–590.

Rossdale PD (1993) Clinical view of disturbances in equine foetal maturation. *Equine Vet. J. Suppl.* **14**, 3–7.

Rumessen JJ, Peters S, Thuneberg L (1993) Light- and electron microscopical studies of interstitial cells of Cajal and muscle cells at the submucosal border of human colon. *Lab. Invest.* **68**, 481–494.

Rumessen JJ (1996) Ultrastructure of interstitial cells of Cajal at the colonic submuscular border in patients with ulcerative colitis. *Gastroenterology* **111**, 1147–1155.

Sanders KM, Stevens R, Burke EP, Ward SM (1990) Slow waves actively propagate at submucosal surface of circular layer in canine colon. *Am. J. Physiol.* **259**, G258–G263.

Sanders KM (1996) A case for interstitial cells of Cajal as pacemakers and mediators of neurotransmission in the gastrointestinal tract. *Gastroenterology* **111**, 492–515.

Smith TK, Reed JB, Sanders KM (1987) Origin and propagation of electrical slow waves in circular muscle of canine proximal colon. *Am. J. Physiol.* **252**, C215–C224.

Thuneberg L (1982) Interstitial cells of Cajal: intestinal pacemaker cells. *Adv. Anat. Embryol. Cell Biol.* **71**, 1–130.

Torihashi S, Ward SM, Nishikava S-I, Nishi K, Kobayashi S, Sanders KM (1995) C-kit-dependent development of interstitial cells and electrical activity in the murine gastrointestinal tract. *Cell Tissue Res.* **280**, 97–111.

Torihashi S, Ward SM, Sanders KM (1997) Development of c-kit-positive cells and the onset of electrical rhythmicity in murine small intestine. *Gastroenterology* **112**, 144–155.

Torihashi S, Nishi K, Tokutomi Y, Nishi T, Ward SM, Sanders KM (1999) Blockade of kit signaling induces transdifferentiation of interstitial cells of Cajal to a smooth muscle phenotype. *Gastroenterology* **117**, 140–148.

Vaala WE (1994) Peripartum asphyxia. *Vet. Clin. North Am.* **10**, 187–218.

Wang XY, Sanders KM, Ward SM (1999) Intimate relationship between interstitial cells of Cajal and enteric nerves in the guinea-pig small intestine. *Cell Tissue Res.* **295**, 247–256.

Wang XY, Sanders KM, Ward SM (2000) Relationship between interstitial cells of Cajal and enteric motor neurons in the murine proximal colon. *Cell Tissue Res.* **302**, 331–342.

Ward SM, Sanders KM (1990) Pacemaker activity in septal structures of canine colonic circular muscle. *Am. J. Physiol.* **258**, G264–G273.

Ward SM, Keller R, Sanders KM (1991) Structure and organization of the electrical activity of the canine distal colon. *Am. J. Physiol.* **260**, G724–G735.

Ward SM, Burns AJ, Torihashi S, Sanders KM (1994) Mutation of the proto-oncogene *c-kit* blocks development of interstitial cells and electrical activity in the murine gastrointestinal tract. *J. Physiol.* **480**, 91–97.

Ward SM, Harney SC, Bayguyinov JR, McLaren GJ, Sanders KM (1997) Development of electrical rhythmicity in the murine

gastrointestinal tract is specifically encoded in the tunica muscularis. *J. Physiol.* **505**, 241–258.

Wester T, Eriksson L, Olsson Y, Olsen L (1999) Interstitial cells of Cajal in the human fetal small bowel as shown by c-kit immunohistochemistry. *Gut* **44**, 65–71.

Wilson JH, Cudd TA (1990) Section three: common gastrointestinal diseases. In *Equine Clinical Neonatology* (eds Koterba AM, Drummond WH, Kosch PC), pp. 412–430. Malvern, PA: Lea & Febiger.

Yamataka A, Ohshiro K, Kobayashi H, Lane GL, Yamataka T, Fujiwara T, Sunagawa M, Miyano T (1998) Abnormal distribution of intestinal pacemaker (c-kit)-positive cells in an infant with chronic idiopathic intestinal pseudoobstruction. *J. Ped. Surg.* **33**, 859–862.

Young HM, Ciampoli D, Southwell BR, Newgreen DF (1996) Origin of the interstitial cell of Cajal in the mouse intestine. *Dev. Biol.* **180**, 97–107.

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Interstitial cells of Cajal (ICC) in equine colic: an immunohistochemical study of horses with obstructive disorders of the small and large intestines

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Keywords: horse; colic; immunohistochemistry; interstitial cells of Cajal

Summary

Reasons for performing study: **The gastrointestinal pacemaker cells, the interstitial cells of Cajal (ICC), have been implicated in several human gastrointestinal dysmotility syndromes. Recently, the involvement of these cells in equine gastrointestinal diseases has been investigated in cases of equine grass sickness where a significant reduction in ICC density was observed.**

Objective: **To investigate ICC density in equine obstructive gastrointestinal disorders using immunohistochemical labelling methods.**

Methods: **Intestinal samples were analysed from 44 horses undergoing exploratory surgery for colic and from 11 control animals subjected to euthanasia for conditions not related to the gastrointestinal tract. Immunohistochemical labelling of ICC was carried out using an anti-c-Kit antibody. Two independent observers assessed ICC density using a semi-quantitative grading system.**

Results: **There was a significant reduction in ICC density in horses with large colon disorders compared to the controls ($P<0.01$). Horses with strangulating lesions of the small intestine showed no difference when compared to the controls.**

Conclusions: **There was a reduction in ICC density in horses with large intestinal disorders.**

Potential relevance: **The reduction in ICC density may be associated with the clinical findings as well as recurrent colic episodes observed in a number of these cases. This immunohistochemical study provides a basis for future functional electrophysiological investigations to determine the precise effect of ICC reduction on equine intestinal motility.**

Introduction

Gastrointestinal motility disorders constitute a significant cause of morbidity and mortality in the horse (Tinker *et al.* 1997) but underlying causes are not accurately determined in a large proportion of cases. This could be due partly to the fact that many colic episodes resolve spontaneously or with medical treatment (Hillyer *et al.* 2001). For those horses undergoing exploratory surgery, the cause of the lesion may or may not be evident at surgery. Clearly, some strangulating conditions of the small intestine have a well-defined physical cause, such as a pedunculated lipoma. However, other conditions, such as large colon displacements or torsions as well as simple obstructions and impactions, may have an underlying cause that is less obvious, for example, due to an abnormal motility pattern. Some horses with acute and chronic obstructive disorders have been shown to have significantly reduced myenteric neuron densities in the large colon or caecum when compared to normal horses (Schusser and White 1997; Schusser *et al.* 2000). It was suggested that these changes may be a reason for the increased risk of recurrent colic from colonic or caecal dysfunction.

The gastrointestinal pacemaker cells, the interstitial cells of Cajal (ICC), have recently been identified in the horse (Hudson *et al.* 1999). These cells are responsible for initiating the slow wave activity in the gastrointestinal tract that is integral to the coordination of gastrointestinal motility (Bortoff 1965; Taylor *et al.* 1975; Duthie and Kirk 1978; Sanders and Smith 1986; Maeda *et al.* 1992; Sanders 1996; Ward *et al.* 1997; Sanders *et al.* 1999). Additional suggested functions of the ICC include mediation of neurotransmission and active propagation of electrical events (Thuneberg 1982; Rumessen *et al.* 1993; Rumessen 1996; Torihashi *et al.* 1997; Wang *et al.* 1999, 2000).

Progress in understanding the function of ICC was made when it was discovered that the proto-oncogene c-Kit encodes a receptor tyrosine kinase, c-Kit, in these cells (Maeda *et al.* 1992). Blocking of this receptor with neutralising antibodies resulted in reduced c-Kit immunoreactivity and abnormal contractile patterns in intestinal muscles with loss of slow wave activity (Maeda *et al.* 1992).

The successful identification of ICC using c-Kit immunohistochemistry has resulted in extensive research into the involvement of these cells in human gastrointestinal motility disorders. A reduction in ICC density has been implicated in human dysmotility syndromes including slow transit constipation (He *et al.* 2000; Knowles and Martin 2000), chronic idiopathic intestinal pseudo-obstruction (Isozaki *et al.* 1997), megacolon (Faussone-Pellegrini *et al.* 1999), Chagasic megacolon (Hagger *et al.* 2000) and ulcerative colitis (Rumessen 1996).

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TABLE 1: Samples collected from 44 clinical cases

Diagnosis	No. cases	Anatomical site
Lipoma	5	Jejunum (3)/ileum (2)
EFE	5	Jejunum (1)/ileum (4)
LCA	28	Pelvic flexure
PLCI	6	Pelvic flexure

Lipoma = Strangulating lipoma; EFE = Epiploic foramen entrapment; LCA = Large colon accident; PLCI = Primary large colon impaction.

A significant reduction in ICC density has been found in horses with grass sickness (Hudson *et al.* 2001). The possible effect on intestinal motility in these cases was demonstrated by observation *in vitro* of reduced slow wave frequency in the diseased ileum compared to normal control animals (Hudson *et al.* 2002).

All of these above mentioned changes in the ICC population have been identified using c-Kit immunohistochemistry. The current study was carried out, using a similar method of identification, in order to investigate if a reduction of ICC density was present in certain obstructive disorders of the equine intestine.

Materials and methods

Animals

Intestinal samples from 11 control horses were collected immediately (within 1 h) following euthanasia by i.v. administration of quinalbarbitone sodium BP (400 mg/ml) and cinchocaine hydrochloride BP (Somulose)[1] (25 mg/ml). The collected samples included ileum (level with the midpoint of the ileocaecal fold) and pelvic flexure apex from each animal. All control horses were killed due to conditions not involving the gastrointestinal tract, e.g. orthopaedic disease and none had a known history of colic.

Intestinal samples were also collected from 44 horses undergoing exploratory laparotomy for colic. Ten of these horses had a strangulating obstruction of the small intestine caused by either epiploic foramen entrapment (n = 5) or by a pedunculated lipoma (n = 5) (Table 1). Samples collected from this group included sections of jejunum or ileum from the nonischaemic tissue at the margin of resection. Although an attempt was made to select tissue that was closest to or included ileum to match the control samples, this was sometimes not possible if the strangulating lesion involved jejunum only (n = 4) (Table 1). The remaining 34 horses had a large colon disorder comprising primary large colon impaction (PLCI) (n = 6) or large colon accident (LCA) involving large colon displacement or torsion (n = 28) (Table 1). Three cases in this group were excluded from the study based on haematoxylin and eosin evaluation (see below). The samples evaluated were all surplus tissue following a pelvic flexure apex biopsy submitted for histological analysis collected during an enterotomy procedure prior to large colon evacuation. All PLCI, of which 5 were sand impactions, as well as three left dorsal colon displacements were refractory to initial medical treatment. On the basis of deteriorating clinical parameters and poor response to analgesic administration, initial medical treatment was not considered appropriate in the remaining animals. As these horses were admitted to referral hospitals, and it is recognised that there may be a bias towards more severely affected animals in this study.

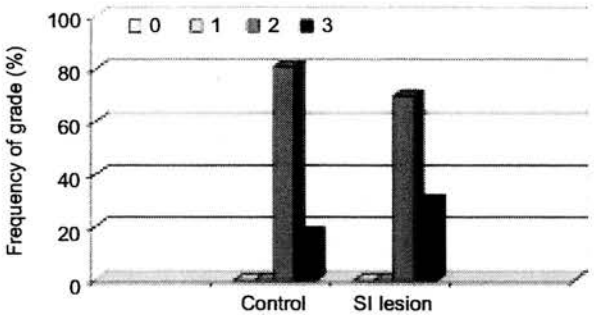

Fig 1: Combined ICC densities of the small intestine (SI) of control horses (n = 11) and those with a strangulating SI lesion (n = 10). Frequency of grade (%) represents the proportion of specimens assigned to a particular density grade: 0 = absent; 1 = sparse; 2 = moderate; 3 = abundant.

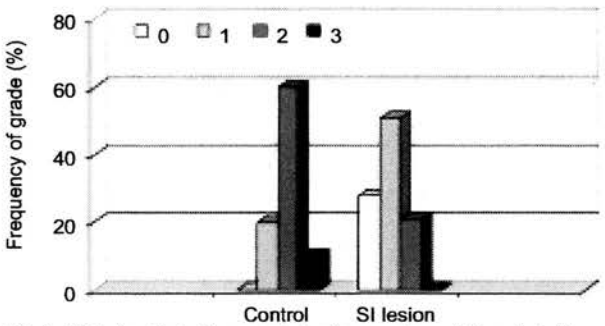

Fig 2: ICC density in the myenteric plexus region of the pelvic flexure region of control horses (n = 11) and those with a large intestine obstructive disorder (n = 31). Frequency grade (%) represents the proportion of specimens assigned to a particular density grade: 0 = absent; 1 = sparse; 2 = moderate; 3 = abundant.

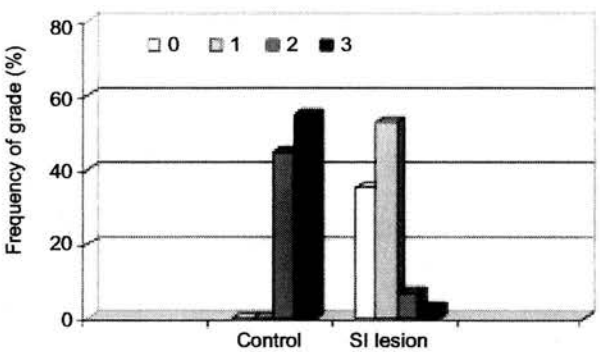

Fig 3: ICC density in the circular muscle region of the pelvic flexure region of control horses (n = 11) and those with a large intestine obstructive disorder (n = 31). Frequency grade (%) represents the proportion of specimens assigned to a particular density grade: 0 = absent; 1 = sparse; 2 = moderate; 3 = abundant.

Sixteen of the 34 animals in this category had a history of previous colic episodes and 4 of these horses had undergone previous surgery to correct the same condition (one PLCI, one recurrent colic without an obvious lesion at surgery and 2 large colon displacements). All samples used in this study were collected with the owners' written consent.

Sample collection and preparation

Following collection, samples were placed immediately in 10% phosphate-buffered formalin and fixed for at least 24 h. After

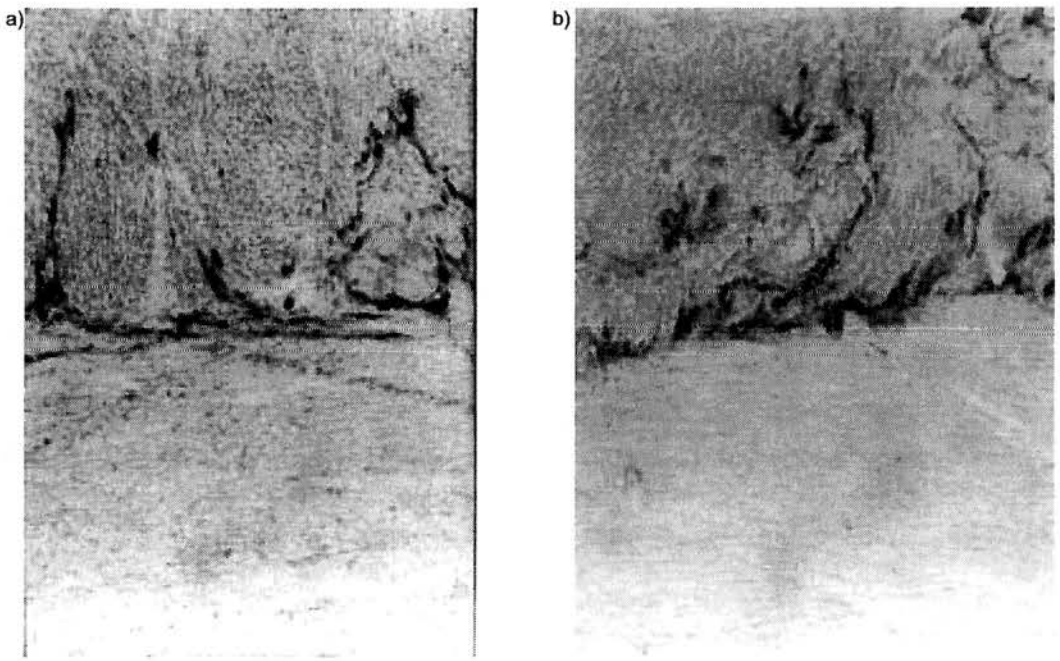

Fig 4: Section of the myenteric region of the ileum of a control animal (a) and that of a horse with a strangulating lesion (b). There is little difference in the degree of immunoreactivity in the myenteric plexus region between these 2 samples. An ICC density grade of 2 (moderate) would have been assigned to both these samples (original magnification x75).

rinsing the tissue samples in tap water, the tissues were cryo-protected in graded sucrose solutions overnight (10 and 30%, respectively) before being frozen rapidly in isopentane pre-cooled in dry ice. Before sectioning, the tissue blocks were orientated to ensure the circular muscle layer of the *muscularis externa* was cut parallel to the fibre direction. This orientation allowed better visualisation of ICC as well as standardising the method. Cryostat sections were cut sequentially at thicknesses of 10 and 20 µm at 500 µm intervals. All tissues were mounted on Tespa (3-aminopropyltriethoxysilane)[2]-coated slides and allowed to air-dry overnight. The sections were then incubated for 30 mins in 0.3% hydrogen peroxide in methanol to quench endogenous peroxidase activity. Nonspecific antibody binding was blocked by a 60 mins incubation in 1% goat serum[3] in phosphate-buffered saline (PBS). Sections were then incubated overnight at 4°C in humid chambers in a rabbit-raised polyclonal antiserum to c-Kit (Ab-1)[4] at a concentration of 1 µg/ml. After washing with PBS, the sections were incubated for 60 mins at room temperature in a biotin-conjugated goat anti-rabbit immunoglobulin at a concentration of 1:200 (Ab-2)[3]. Immunoreactivity was revealed using the avidin-biotin (ABC)[5] method using a diaminobenzidine substrate (DAB)[6]. Sections were dehydrated in ethanol, cleared in xylene and then mounted in Depex[7].

Negative controls were prepared in a similar manner except that the primary antibody was replaced with normal rabbit serum. This resulted in complete absence of immunolabelling. Positive controls were performed using tissue from a normal control animal from a previous study that had been shown to have abundant c-Kit immunoreactivity. Both positive and negative controls were included for every batch of samples processed.

The immunohistochemical labelling was assessed semi-quantitatively based on similar grading techniques described in earlier studies (Hagger *et al.* 1998a,b, 2000; Hudson *et al.* 2001). In the study by Hudson *et al.* (2001), the absence of c-Kit immunoreactivity was graded 0, sparse 1, moderate 2 and abundant 3. This grading system was adopted for this study.

In addition to the immunohistochemical labelling, all processed tissues were stained with haematoxylin and eosin (H&E) in order to assess tissue integrity. This was carried out in order to help clarify whether reduced c-Kit immunoreactivity was a primary finding or secondary to a degree of tissue autolysis. Only samples with good tissue integrity (and not worse than a mild degree of mucosal degradation) were included to ensure that immunohistochemical grading was reliable. Three animals with large colon torsions were rejected from the study on this basis taking the total number of colic cases included in this study to 41.

Statistical analysis

ICC density grades of normal and all colic horses as well as horses with a history of recurrent colic episodes and horses without a known history of colic involving the large colon were compared using the Mann-Whitney test. Separate evaluation of the myenteric and circular muscle regions as well as combined c-Kit immunoreactivity was carried out for all samples. A significance level of 5% was assumed for all tests.

Two independent observers assessed and graded all tissue sections. The grading of each animal was based on the scrutiny of at least 4 adjacent 10 µm sections of tissue. In the event of disagreement of classification between the 2 observers, a consensus grade was assigned after a joint review of the sections. A Mann-Whitney test assuming a significance level of 5% was used to evaluate interobserver variability.

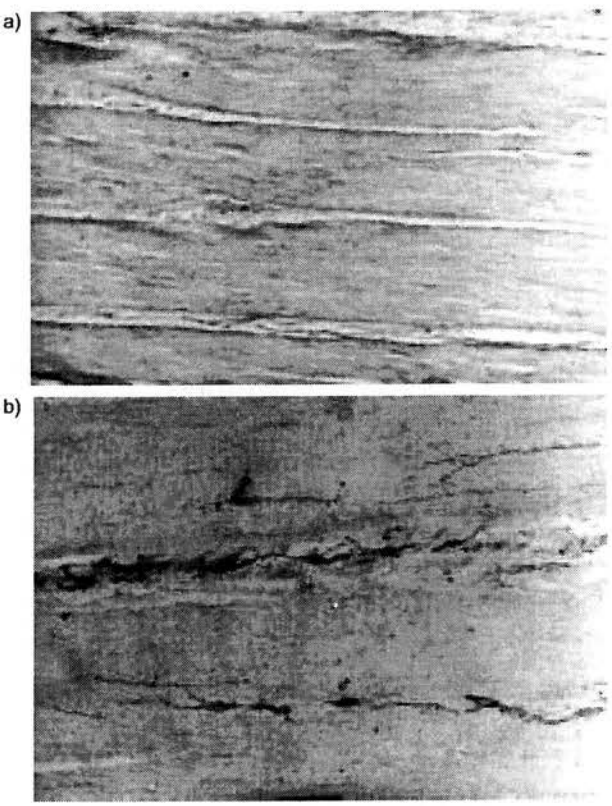

Fig 5: Section of the circular muscle of the pelvic flexure of a) a control animal and b) a horse with a large colon torsion. There is a reduced degree of immunoreactivity in the affected animal where a grade 2 and 0, respectively, would have been assigned to these samples (original magnification x75).

Results

Animals

The control animals included 6 geldings and 5 mares, median age 12 years (mean 11.7 years, range 1–33 years). The colic group consisted of 21 geldings, 19 mares and 1 stallion, median age 12 years (mean 11.9 years, range 1–28 years). The horses affected with small intestinal lesions consisted of 1 stallion, 6 geldings and 3 mares, median age 10 years (mean 13.4 years, range 1–28 years). The horses with large colon disorders consisted of 16 mares and 15 geldings, median age 11 years (mean 11.5 years, range 1–24 years).

Immunohistochemical and semi-quantitative ICC evaluation and H&E evaluation

Immunohistochemical labelling of c-Kit indicated the presence of ICC in both control and surgical colic cases. There was no significant difference between the combined myenteric and circular muscle region ICC densities in small intestinal samples from surgical colic cases and from control horses (Fig 1). In addition, there was no significant difference in ICC densities in either myenteric region (Fig 4) or circular muscle layer of the small intestine between the 2 groups when comparing these anatomical areas separately.

In contrast, combined ICC densities were reduced significantly in horses with large colon obstructive disorders compared to control animals ($P<0.01$). This difference was also significant when myenteric region ($P<0.01$) and circular muscle layer ($P<0.01$) ICC densities were evaluated separately (Figs 2, 3 and 5). There was no significant difference in the myenteric region, the circular muscle layer or overall ICC densities in horses with a history of recurrent colic episodes compared to horses without a known history of colic. There was no statistical difference between the grading of the 2 independent observers.

In 5 cases with a strangulating obstruction of the small intestine, c-Kit immunoreactivity was examined also in the grossly compromised segment of small intestine. Reduced ICC density was noted compared to the nonischaemic tissue at the margin of resection from the same animal. However, based on the degree of tissue degradation assessed by H&E stain, these samples were not included in the statistical analysis.

Evaluation of the H&E sections of the small intestine from affected horses revealed variable mild to moderate lymphocytic infiltration of the mucosa and submucosa which was not evident in the *muscularis externa*. Large colon samples from affected horses also revealed mild to moderate lymphocytic infiltration of the mucosa and submucosa. In some areas of the *muscularis externa*, there was a mild perivascular lymphocytic infiltrate. This infiltrate did not extend beyond the perivascular connective tissue. An occasional migrating neutrophil was also observed in the *muscularis externa*. In addition, there was no obvious infiltrate in the region of the myenteric plexus region in either the small or large intestine samples.

Discussion

Our findings demonstrate a reduction in the extent of ICC density in pelvic flexure samples from animals with a large colon disorder when compared to the controls. It is possible that the reduced ICC density may already have been present prior to surgical correction. This begs the question whether the colic episode necessitating surgical correction was a consequence of, rather than the cause of the reduced ICC density. It is more likely that the reduction in ICC density was a possible cause of the colic rather than an effect. Sixteen of the 31 animals in this category had a history of colic episodes and 4 of these horses had undergone previous surgical correction for the same condition. Previous studies have demonstrated that some horses suffering from one bout of colic are more likely to suffer further bouts (Cohen *et al.* 1995; Tinker *et al.* 1997; French *et al.* 2002). There was no difference statistically in ICC densities in horses with a history of recurrent colic episodes compared to those without a known history of previous colic. However, given the reduction of ICC in the total colic population compared to control animals, it is possible that the colic was the result of reduced ICC density as it is known from previous studies that alterations in ICC density affect slow wave activity which will also influence intestinal motility patterns (Maeda *et al.* 1992; Hudson *et al.* 2002). Hudson *et al.* (2002) demonstrated that slow wave activity was still present in horses with an observed reduction of ICC numbers in equine grass sickness although at reduced frequency. This could support the suggestion that recovered grass sickness horses may be more prone to intestinal motility disorders and hence more sensitive to management changes (Doxey *et al.* 1995).

Little is known about the processes that lead to the loss of ICC in clinical disorders of gastrointestinal motility (Sanders *et al.*

1999). In our equine study, it is less likely that the reduction in ICC density was a consequence of the colic episode. The timescale involved in these colic cases would appear to be too short to cause ICC loss resulting in disrupted motility. In an experimental model of murine intestinal pseudo-obstruction (a surgically-applied partial occlusion clip around the intestine), ICC were not affected 48–72 h after administration of the clips, but were diminished by 14 days (Chang *et al.* 2001).

Both hypoxia and inflammation have been implicated as possible causes of damage to ICC. Christensen and Rick (1994) suggested that ICC were susceptible to hypoxia by demonstrating an increase in intracellular vacuolation in response to hypoxia. Lu *et al.* (1997) demonstrated that inflammation damaged ICC in canine colon resulting in alteration of slow wave characteristics that contributed to the suppression of phasic contractions under *in vitro* conditions. It is possible that some degree of hypoxia and associated inflammation took place during the pathological processes of intestinal obstruction or as a consequence of tissue handling during surgery (Fabri and Rosemurgy 1991). However, it is unlikely that this caused the observed reduction in ICC density again because the time course would have been too short. It has been shown that it takes at least 18 h for inflammation to cause disruption of ICC (Der *et al.* 2000). This would suggest that tissue handling and possible hypoxia at surgery are highly improbable explanations for our results. Additionally, the presence of a very mild degree of inflammatory cell infiltrate observed in the *muscularis externa* would further support this. Indeed, the surgical small intestinal samples showed no reduction in ICC density compared to the controls and these samples would have had much greater tissue handling (e.g. manual decompression) than the large colon samples where an ICC reduction was observed.

It is recognised that in 4 of the 10 cases involving sections of small intestine, jejunum rather than ileum was evaluated due to the anatomical location of the strangulating lesion. Although this differed from the location of the controls (ileum), an effort was made always to evaluate the most distal segment of jejunum. Additionally, Hudson *et al.* (1999) described a similar pattern of ICC distribution and level of expression in these two areas despite the fact that there appeared to be particularly strong immunoreactivity in the ileum. Considering the presence of strong immunoreactivity and a similar distribution of ICC in the jejunum of affected animals compared to control animals, it was considered a valid comparison despite the anatomical variation.

This immunohistochemical study has demonstrated a reduction in the intestinal pacemaker cell (ICC) density in horses with disorders of the large colon compared to control animals. Normal intestinal motility patterns involve a closely integrated process involving intestinal smooth muscle, nervous input as well as the ICC and alteration of any of these components may affect normal motility patterns. The precise nature of the effect that reduced ICC numbers has on intestinal motility in the horse may only be obtained through further electrophysiological and myoelectrical studies. This would help in expanding our knowledge of equine intestinal disorders as these not only cause discomfort and suffering to affected animals but also represent significant annual economical losses (Traub-Dargatz *et al.* 2001).

Acknowledgements

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Manufacturers' addresses

[1]Arnolds Veterinary Products, ?, UK.
[2]Sigma, Poole, Dorset, UK.
[3]Vector Laboratories, Burlingame, California, USA.
[4]Oncogene Research Products, Cambridge, Massachusetts, USA.
[5]Vector Laboratories, Burlingame, California, USA.
[6]BDH Laboratory Supplies, Poole, Dorset, UK.
[7]Merck, Glasgow, UK.

References

Bortoff, A. (1965) Electrical transmission of slow waves from longitudinal to circular intestinal muscle. *Am. J. Physiol.* **209**, 1254-1260.

Chang, I., Glasgow, N.J., Takayama, I., Horiguchi, K., Sanders, K.M. and Ward, S.M. (2001) Loss of interstitial cells of Cajal and development of electrical dysfunction in murine small bowel obstruction. *J. Physiol.* **536**, 555-568.

Christensen, J. and Rick, G.A. (1994) Interstitial cells of Cajal in the rat colon are damaged by mild hypoxia. *J. autonom. Nerv. Syst.* **48**, 175-180.

Cohen, N.D., Matejka, P.L., Honnas, C.M. and Hooper, R.N. (1995) Case-control study of the association between various management factors and development of colic in the horse. *J. Am. vet. med. Ass.* **206**, 667-673.

Der, T., Bercik, P., Donnelly, G., Jackson, T., Berezin, I., Collins, S.M. and Huizinga, J.D. (2000) Interstitial cells of Cajal and inflammation-induced motor dysfunction in the mouse small intestine. *Gastroenterol.* **119**, 1590-1599.

Doxey, D.L., Tothill, S., Milne, E.M. and Davies, Z. (1995) Patterns of feeding and behaviour in horses recovering from dysautonomia (grass sickness). *Vet. Rec.* **137**, 181-183.

Duthie, H.L. and Kirk, D. (1978) Electrical activity of human colonic smooth muscle *in vitro*. *J. Physiol.* **283**, 319-330.

Fabri, P.J. and Rosemurgy, A. (1991) Reoperation for small intestinal obstruction. *Surg. Clin. N. Am.* **71**, 131-146.

Faussone-Pellegrini, M.S., Fociani, P., Buffa, R. and Basilisco, G. (1999) Loss of interstitial cells and a fibromuscular layer on the luminal side of the colonic circular muscle presenting as megacolon in the adult patient. *Gut* **45**, 775-779.

French, N.P., Smith, J., Edwards, G.B. and Proudman, C.J. (2002) Equine surgical colic: risk factors for postoperative complications. *Equine vet. J.* **34**, 444-449.

Hagger, R., Gharaie, S., Finlayson, C. and Kumar, D. (1998a) Regional and transmural density of interstitial cells of Cajal in human colon and rectum. *Am. J. Physiol.* **275**, G1309-G1316.

Hagger, R., Gharaie, S., Finlayson, C. and Kumar, D. (1998b) Distribution of the interstitial cells of Cajal in the human anorectum. *J. autonom. Nerv. Syst.* **73**, 75-79.

Hagger, R., Finlayson, C., Kahn, F., De Oliveira, R., Chimelli, L. and Kumar, D. (2000) A deficiency of interstitial cells of Cajal in Chagasic megacolon. *J. autonom. Nerv. Syst.* **80**, 108-111.

He, C.-L., Burgart, L., Wang, L., Pemberton, J., Young-Fadok, T., Szurszewski, J. and Farrugia, G. (2000) Decreased interstitial cell of Cajal volume in patients with slow-transit constipation. *Gastroenterol.* **118**, 14-21.

Hillyer, M.H., Taylor, F.G.R. and French, N.P. (2001) A cross-sectional study of colic in horses on Thoroughbred training premises in the British Isles in 1997. *Equine vet. J.* **33**, 380-385.

Hudson, N.P.H., Mayhew, I.G. and Pearson, G.T. (2001). A reduction in interstitial cells of Cajal in horses with equine dysautonomia (grass sickness). *Autonom. neurosci. Basic Clin.* **92**, 37-44.

Hudson, N.P.H., Mayhew, I.G. and Pearson, G.T. (2002) Presence of *in vitro* electrical activity in the ileum of horses with enteric nervous system pathology: equine dysautonomia (grass sickness). *Autonom. neurosci. Basic Clin.* **99**, 119-126.

Hudson, N.P.H., Pearson, G.T., Kitamura, N. and Mayhew, I.G. (1999) An immunohistochemical study of interstitial cells of Cajal in the equine gastrointestinal tract. *Res. vet. Sci.* **66**, 265-271.

Isozaki, K., Hirota, S., Miyagawa, J.-I., Taniguchi, M., Shinomura, Y. and Matsuzawa, Y. (1997) Deficiency of c-kit$^+$ cells in patients with a myopathic form of chronic idiopathic intestinal pseudo-obstruction. *Am. J. Gastroenterol.* **92**, 332-334.

Knowles, C.H. and Martin, J.E. (2000) Slow transit constipation: A model of human gut dysmotility. Review of possible actiologics. *Neurogastroent. Motil.* **12**, 181-196.

Lu, G., Qian, X., Berezin, I., Telford, G.L., Huizinga, J.D. and Sarna, S.K. (1997) Inflammation modulates *in vitro* colonic myoelectric and contractile activity and interstitial cells of Cajal. *Am. J. Physiol.* G1233-G1245.

Maeda, H., Yamagata, A. and Nishikawa, S. (1992) Requirement of c-kit for development of intestinal pacemaker system. *Development* **116**, 369-375.

Rumessen, J.J. (1996) Ultrastructure of interstitial cells of Cajal at the colonic submucosal border in patients with ulcerative colitis. *Gastroenterol.* **111**, 1447-1455.

Rumessen, J.J., Mikkelsen, H.B., Qvortrup, K. and Thuneberg, L. (1993) Ultrastructure of interstitial cells of Cajal in circular muscle of human small intestine. *Gastroenterol.* **104**, 343-350.

Sanders, K.M. (1996) A case for interstitial cells of Cajal as pacemakers and mediators of neurotransmission in the gastrointestinal tract. *Gastroenterol.* **111**, 492-515.

Sanders, K.M. and Smith, T.K. (1986). Motorneurones of the submucous plexus regulate electrical activity of the circular muscle of canine proximal colon. *J. Physiol.* **380**, 293-310.

Sanders, K.M., Ördog, T., Koh, S.D. and Ward, S.M. (1999) Development and plasticity of interstitial cells of Cajal. *Neurogastroent. Motil.* **11**, 311-338.

Schusser, G.F. and White, N.A. (1997) Morphologic and quantitative evaluation of the myenteric plexus and neurons in the large colon of horses. *J. Am. vet. med. Ass.* **210**, 928-934.

Schusser, G.F., Scheidemann, W. and Huskamp, B. (2000) Muscle thickness and neuron density in the caecum of horses with chronic recurrent caecal impaction. *Equine vet. J., Suppl.* **32**, 69-73.

Taylor, I., Duthie, H.L., Smallwood, R. and Linkens, D. (1975) Large bowel myoelectrical activity in man. *Gut* **16**, 808-814.

Thuneberg, L. (1982) Interstitial cells of Cajal: Intestinal pacemaker cells. *Adv. Anat. Embr. Cell Biol.* **71**, 1-130.

Tinker, M.K., White, N.A., Lessard, P., Thatcher, C.D., Peltzer, K.D., Davis, B. and Carmel, D. (1997) A prospective study of equine colic incidence, risk factors and mortality rates. *Equine vet. J.* **29**, 448-453.

Torihashi, S., Ward, S.M. and Sanders, K.M. (1997) Development of c-kit-positive cells and the onset of electrical rhythmicity in murine small intestine. *Gastroenterol.* **112**, 144-155.

Traub-Dargatz, J.L., Kopral, C.A., Seitzinger, A.H., Garber, L.P., Forde, K. and White, N.A. (2001) Estimate of the national incidence of and operation-level risk factors for colic among horses in the United States, spring 1998 to spring 1999. *J. Am. vet. med. Ass.* **219**, 67-71.

Wang, X.Y., Sanders, K.M. and Ward, S.M. (1999) Intimate relationship between interstitial cells of Cajal and enteric nerves in the guinea-pig small intestine. *Cell Tissue Res.* **295**, 247-256.

Wang, X.Y., Sanders, K.M. and Ward, S.M. (2000) Relationship between interstitial cells of Cajal and enteric motor neurons in the murine proximal colon. *Cell Tissue Res.* **302**, 331-342.

Ward, S.M., Harney, S.C., Bayguinov, J.R., McLaren, G.J. and Sanders, K.M. (1997) Development of electrical rhythmicity in the murine gastrointestinal tract is specifically encoded in the *tunica muscularis*. *J. Physiol.* **505**, 241-258.

There was a trend of increasing rates of confirmed adhesions with increasing total protein concentration at admission. The rate of adhesion formation was highest in horses that had strangulating small bowel obstructions. The rate of confirmed adhesions was also significantly higher in horses that had a bowel resection, repeat laparotomy and cases that developed post operative ileus. Colic occurred after discharge in 27.7%. The rate of colic after discharge was higher in horses with small bowel obstructions, thoses that had bowel resected and those that developed post operative ileus. The death rate was higher in horses that had a history of colic after discharge (25.9%) compared to those that did not (11.9%). Ventral hernia formation was recorded at follow-up in 8.1%. The rate of ventral hernia formation was highest in horses that developed post operative wound drainage or sepsis.

[10.00–10.15]

A STUDY INTO RISK FACTORS AND PREVALENCE OF POST OPERATIVE COLIC AFTER ORTHOPAEDIC SURGERY

Senior J.M., Pinchbeck G., Dugdale A.H.A. and Clegg P.D. (CHESHIRE, UK)

A retrospective study was conducted to estimate the prevalence of, and identify risk factors for, the development of post anaesthetic colic in horses undergoing orthopaedic surgery at our hospital. Colic was defined as any recognised symptom of abdominal pain that could not be attributed to any concurrent disease. We hypothesised that in hospitalised horses there is an increased risk of colic due to dietary and management changes, as well as the administration of drugs such as opioids, NSAIDs, sedative and anaesthetic agents, which may compromise gastrointestinal function.

Materials and Methods: Records for a total of 496 anaesthetics in 428 horses were recorded. We estimated a prevalence of at least 5%. This would give 80% power with 95% confidence to detect risk factors with an odds ratio of 3.0 or more for exposures of 25–50%.

Results: There was a prevalence of post operative colic of 2.8%. The majority of colics (69%) were undiagnosed, 12% were classified as impactions and 25% as tympanic colic.

The risk of post operative colic was associated with the use of opioids. When opioids were divided into types (butorphanol and morphine), morphine was associated with a 4-fold increased risk of post operative colic compared to the use of no opioid or butorphanol (OR = 4.1, 95% CI = 1.4, 12.2). Out-of-hours surgery was also associated with an increased risk of developing post operative colic (OR = 3.0, 95% CI = 1.0, 8.8)

In conclusion, this study has provided an estimate of the prevalence of post operative colic in patients undergoing orthopaedic surgery at our clinic and has identified potential risk factors. A further prospective case-control study with greater case numbers is now required to confirm the role of these risk factors while controlling for other potentially confounding factors.

[10.15–10.30]

THE INTERSTITIAL CELLS OF CAJAL (ICC) IN EQUINE COLIC: AN IMMUNOHISTOCHEMICAL STUDY OF HORSES WITH OBSTRUCTIVE DISORDERS OF THE SMALL AND LARGE INTESTINES

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Gastrointestinal (GI) motility disorders constitute a significant cause of morbidity and mortality in the horse. The GI pacemaker cells, the interstitial cells of Cajal (ICC), have recently been identified in the horse. These cells play an integral part in the coordination of normal motility patterns in the GI tract, as they are the initiators of slow wave activity. The ICC also mediate neurotransmission. The effect of changes in the ICC population on GI tract motility patterns is well documented in several mammalian species. A significant reduction in ICC density has also been observed in cases of equine grass sickness. The objective of this study was to investigate ICC density in equine obstructive GI disorders using standard immunohistochemical labelling methods.

Intestinal samples from 41 horses undergoing exploratory surgery for colic were evaluated. This included sections of the jejunum or ileum from 10 horses with a strangulating lesion of the small intestine and sections of the pelvic flexure from 31 horses with large colon lesions that warranted surgical correction. Samples from 11 control animals subjected to euthanasia for conditions not related to the GI tract were also collected. Immunohistochemical labelling of ICC was carried out using an anti-c-Kit antibody and ICC density was assessed using a semiquantitative grading system. Parallel H&E staining was carried out for each section to ensure that tissue quality was unlikely to influence the c-Kit immunoreactivity. Statistical analysis indicated that there was a significant reduction in the overall ICC density in horses with obstructive lesions of the large colon ($P<0.0001$) while there was no difference in horses with strangulating lesions of the small intestine ($P = 0.639$). It is possible that this reduction in ICC density in horses with large colon obstructive disorders may be associated with the observed clinical signs, although functional studies are needed to support this hypothesis.

Harwood CL *et al.* (1998). *J Exp Biol* **201**, 2723–2733.
Hongo K *et al.* (1996). *J Physiol* **491**, 599–606.

This work was supported by NSERC Canada and The University of Leeds.

All procedures accord with current UK legislation

C85

The cardiovascular changes associated with warming and cooling in the turtle, *Trachemys scripta*

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The cardiovascular changes associated with warming and cooling were investigated in seven freshwater turtles, *Trachemys scripta*. Animals were anaesthetised by isoflurane inhalation, a piece of the ventral carapace over the heart was removed with a bone saw, and blood flow probes (Transonic Systems Inc.) were placed around the left aortic arch and left pulmonary artery. The carotid artery was occlusively cannulated, using a PE50 catheter, for blood pressure measurements. Animals were then artificially ventilated until spontaneous ventilation resumed, and allowed to recover for a period of 48 hours. The turtles were warmed to 34 °C, using an infrared 150W heating lamp, and then allowed to cool to 24 °C, both before and 45 min after intrarterial injection of atropine (3 mg kg^{-1}). All experimental animals were humanely killed following the protocol using an overdose of Pentobarbitol (intrarterial injection of 200 mg kg^{-1}).

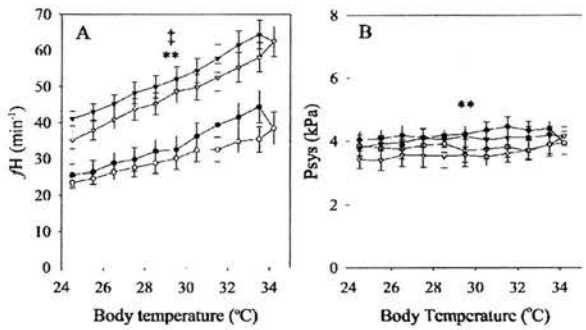

Figure 1. Heart rate (fH) (*A*), and systemic arterial blood pressure (Psys) (*B*), during warming and cooling in untreated (circles) and atropinised (triangles) animals. Values are mean with S.E.M. ($n = 7$). ** Indicates there is a significant difference between warming and cooling; ‡ indicates there is a significant difference between atropinised and untreated animals ($P < 0.05$).

All turtles warmed at a faster rate than they cooled. Animals exhibited a hysteresis of heart rate and blood flow to both the pulmonary and systemic circulations, which was not cholinergically mediated (Fig. 1A). Blood pressure remained constant during both warming and cooling (Fig. 1B), while systemic resistance decreased during heating and increased during cooling, indicating a barostatic response. Hicks (1998) described a large R-L shunt in *Trachemys scripta* which increased with heating and decreased with cooling in untreated animals, interpreting the shunt pattern as a passive consequence of altered resistances within the systemic circulation. However, in the present study, there was a large R-L shunt during warming and cooling in untreated animals which remained relatively constant. This may indicate an active regulation of cardiac shunts in order to sustain oxygen delivery. Atropinisation resulted in a large L-R shunt which decreased during warming and increased during cooling. However, the cooling periods were extended in atropinised animals, indicating that heat lost across the lung surface is not a determining factor in rates of cooling.

Hicks, J.W. (1998). In *Biology of Reptilia, Morphology G. Visceral Organs*, ed. Gans C & Gaunt A.S., pp. 425–483. SSAR Press, Ithaca, NY, USA.

All procedures accord with current UK legislation

C87

The development of the interstitial cells of Cajal in the equine fetus: an immunohistochemical study

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The interstitial cells of Cajal (ICC) are integral to the coordination of gastrointestinal motility by generating pacemaker activity and mediating neurotransmission in the gastrointestinal tract (Sanders 1996). There is currently no information available on the development of the ICC in the equine fetus. This study was carried out in order to determine whether ICC are detectable in the equine fetus, and if so, whether there are age-related changes in the patterns of ICC distribution, with respect to both the transmural and rostro-caudal distribution of ICC.

Tissues from 12 naturally aborted equine fetuses were used in this study. The ages of the fetuses ranged from 6–11 months (normal gestation length in the horse is 11 months). Sections of ileum, caecal base, pelvic flexure and distal small colon were obtained from each animal. All material was collected at post mortem and was surplus to that required for the routine investigation into the cause of abortion. Collected tissue was fixed in formalin and standard immunohistochemical labelling techniques were applied using an antibody against the c-Kit protein of the ICC (Oncogene Research Products, Cambridge, MA, USA).

ICC were present throughout the equine gastrointestinal tract at 6 months of gestation. The distribution of ICC in the small intestine was similar to that of the neonatal animal while in the large intestine changes were observed during development. A rostro-caudal gradient of immunoreactivity was evident, with the more distal part of the large intestine being less densely colonised by ICC in the younger fetuses compared to the near-term animals. A transmural gradient of ICC distribution was also evident within the large intestine, with the most luminal part of the muscularis externa appearing to be the last area of the intestine to be colonised by these cells. Indeed, even in the full term fetus this region did not appear fully developed compared to the neonatal animal. This transmural gradient observed in the horse does not appear to be present in other mammalian species that have been studied. Considering the proposed location of the pacemaker ICC at the submucosal border of the circular muscle layer (Rae *et al.* 1998), this may be of importance when investigating commonly observed problems in the equine neonate such as meconium impactions and motility disorders observed in dysmature and premature foals.

Rae MG *et al.* (1998). *J Physiol* **510**, 309–320.
Sanders KM (1996). *Gastroenterology* **111**, 492–515.

This work was supported by the HBLB.

ments from adjacent sites were identical or very similar in LM and CM: 30–48 per min, 42–60 per min and 60–72 per min in ileum, jejunum and duodenum respectively. These frequencies are significantly higher than those reported for slow waves in stretched, dissected CM pieces of ileum or jejunum. We conclude that LM, like CM probably has slow waves, usually simultaneous with those in CM, but that tonic inhibitory activity in CM may desynchronize the two layers. Also, preparation for intracellular recording by stretching may affect slow wave frequencies.

Acknowledgment
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4

Interstitial cells of Cajal: potential roles in vagal mechanoreceptor development, maintenance and sensory transduction

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The interstitial cells of Cajal (ICC-IMs) are intercalated between neurons and smooth muscle cells, suggesting participation in both motor and sensory activity, and in past research several motor functions have been elaborated. We have conducted a series of experiments in mice to systematically investigate sensory function of the intramuscular class of ICCs (ICC-IMs). Our results suggest that ICC-IMs complex with vagal intramuscular array (IMA) -type mechanoreceptors, potentially to form a sensory transduction module. First, we showed that the terminal processes of vagal IMAs are closely apposed to ICC-IMs in normal mice. Next, to determine whether this apposition might represent a functional relationship, we investigated whether the lack of ICC-IMs in c-Kit mutants was associated with alterations in IMA survival or morphology. Indeed, c-Kit mutants, had substantial reductions in numbers of IMAs, and surviving terminals typically had aberrant morphologies. Importantly, intraganglionic laminar endings, the other class of vagal mechanoreceptors that supply the GI muscle wall, were not affected, suggesting that the effects on IMAs were specific responses to loss of their target cells, rather than non-specific effects on all GI innervation. Then, after determining that mice with mutant steel factor (the ligand for the c-Kit receptor) also lack ICC-IMs, we found these mice had the same IMA effects as c-Kit mutants. Presence of the same altered IMA phenotype in mice with different genetic backgrounds, and distinct mutations that affect the same signaling pathway, strengthens the hypothesis that this altered phenotype results from reduced steel factor-c-Kit signaling and the consequent ICC loss, rather than from other mutations or interactions with background genes. Taken together, our results suggest that ICC-IMs may be necessary for IMA development or maintenance. Therefore, the ICC-IMA complex will provide a valuable model system for investigating nerve terminal – accessory cell interactions in mechanoreceptor development and transduction.

Acknowledgment
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The interstitial cells of Cajal and electrical activity of the equine intestine in health and disease

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Gastrointestinal motility disorders constitute a serious problem in both human and veterinary medicine and may represent a dysfunction of the neural, muscular or pacemaker components (interstitial cells of Cajal, ICC) of bowel control. Equine dysautonomia (grass sickness) is a frequently fatal disease of horses characterised by profound ileus. Colic (the syndrome of abdominal pain) is reported by insurance companies and universities as the single greatest killer of horses. The aims of this work were to investigate the density of ICC and the *in vitro* electrical properties of smooth muscle in healthy and diseased equine intestine. Equine small and large intestinal samples were collected from clinical cases (surgical resections and euthanased animals, from both horses free from gastrointestinal disease and those with grass sickness or obstructive intestinal disorders). ICC were identified using immunohistochemical labelling with an anti-c-Kit antibody and their density assessed using a semi-quantitative grading system. Intracellular microelectrode recordings were made from smooth muscle cells in cross-sectional preparations of ileum, superfused *in vitro*. In horses with grass sickness, ICC were significantly decreased in both the myenteric plexus and circular muscle regions of both ileum and pelvic flexure compared to normal animals. In horses with obstructive lesions of the large colon, ICC were significantly reduced in the myenteric and circular muscle regions. There was no difference in horses with strangulating lesions of the small intestine. Slow waves were recorded in all of the equine ileal preparations from normal horses and from the majority of horses with grass sickness. In comparison to the normal horses, the slow waves in grass sickness horses had a significantly reduced frequency and increased duration. It is possible that the decline in ICC may be an important factor in the development of intestinal dysmotility observed in grass sickness and obstructive bowel disorders in the horse.

6

Nerve-evoked internalization of neurokinin 1 receptors on interstitial cells of Cajal at the deep muscular plexus in the murine small intestine

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Recent studies have shown that ICC are specific targets for neurotransmitters in the stomach. ICC in the small bowel and colon also form tight, synaptic-like contacts with the terminals of enteric motor neurons, but little is known about the role of these cells in neurotransmission. ICC at the deep muscular plexus (ICC-DMP) express NK1 receptors and internalize these receptors in response to exogenous substance P. NK1 receptors are not present in ICC at the level of the myenteric plexus (ICC-MY) in unstimulated tissues but are revealed after exposure to exogenous substance P (SP; Lavin et al., 1998). In the present study we used the internalization of NK1 receptors in ICC-DMP of the murine small intestine (SI) as a means of assessing the degree of innervation of these cells. In the murine SI, NK1 receptor immunoreactivity was limited to ICC-DMP (519 cells counted, all were positive) and myenteric neurons. ICC-DMP were closely apposed to SP-containing nerve fibers. From a total of 338 ICC-DMP examined, 64.9% of cells were associated with one SP-positive nerve fiber, 32.3% were associated with 2 SP nerve fibers, and 1.18% were associated with more than 2 nerve fibers. We were unable to find a clear association with a SP nerve fiber in less than 1% of the ICC-DMP examined. After EFS (10 Hz for 1 min, 0.5 ms pulse duration) NK1 receptors were internalized into ICC-DMP in over 80 % of cells, as compared to 10% of cells prior to EFS ($P < 0.05$). Internalization of NK1 receptors was not observed in ICC-MY in response to nerve stimulation. These data suggest that ICC-DMP are primary targets for neurally released takykinnins in the SI. Exogenous SP may activate receptors that are not normally exposed to SP released from nerves.

Acknowledgment
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7

Interrelationships between spontaneous motility and interstitial cells of Cajal in gut-like clusters from embryonic stem cells

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Using an embryoid body (EB) culture system, we made a functional organ-like cluster, the "gut" from embryonic stem (ES) cells according

The interstitial cells of Cajal and electrical activity of the equine intestine in health and disease

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INTRODUCTION

Gastrointestinal motility disorders constitute a serious problem in both human and veterinary medicine and may represent a dysfunction of the neural, muscular or pacemaker components (interstitial cells of Cajal, ICC) of bowel control. Equine dysautonomia (grass sickness) is a frequently fatal disease of horses characterised by profound ileus (Figures 1 & 2) and severe neuronal lesions in the enteric nervous system. Colic (the syndrome of abdominal pain. Figure 3) is reported by insurance companies and universities as the single greatest killer of horses. The aims of this work were to investigate the density of ICC and the *in vitro* electrical properties of smooth muscle in healthy and diseased equine intestine.

Figure 1. A horse with chronic grass sickness

Figure 2. A large colon impaction from a horse with grass sickness

Figure 3. A horse showing signs of severe colic

METHODS

Tissue preparation

Equine small (ileum) and large (pelvic flexure of large colon) intestinal samples were collected from clinical cases (surgical resections and euthanased animals, from both horses free from gastrointestinal disease {24} and those with grass sickness {28} or obstructive intestinal disorders {41}).

Electrophysiology

Cross-sectional preparations of equine ileum were superfused with modified Krebs-Henseleit solution and maintained at 36-37°C for up to 8 hours.[1] Intracellular recordings of membrane potential were made from both longitudinal and circular smooth muscle cells in both normal and grass sickness-affected tissues. Glass microelectrodes were filled with 2M KCl and had resistances in the range 25-50 MΩ.

Immunohistochemistry

Tissues from the ileum and pelvic flexure of horses were fixed and processed for cryostat sectioning (10µm thickness).[2] A polyclonal rabbit-raised antiserum against c-Kit (Oncogene Research Products, Cambridge MA) was used for the immunohistochemical labelling of ICC. The c-Kit immunoreactivity was visualised using the avidin-biotin (ABC) method. ICC density was assessed using a semi-quantitative grading system.

1. Hudson, NPH, Mayhew, IG & Pearson, GT. (2001) *Veterinary Record* 149, 707-711
2. Hudson, NPH, Pearson, GT, Kitamura, N & Mayhew, IG (1999) *Research in Veterinary Science* 66, 265-271

RESULTS

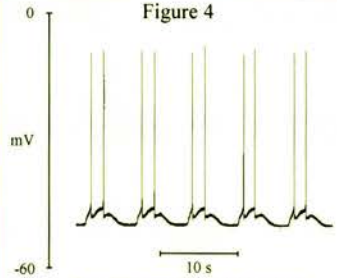
Electrophysiology

- Slow waves recorded in both circular and longitudinal muscle layers (Figs 4,5)
- Nifedipine-sensitive spontaneous action potentials (Fig 4)
- Slow waves recorded in all of the preparations from normal (8) horses (Figs 4,5)
- Slow waves recorded in 4 of 6 grass sickness-affected preparations (Fig 5)
- Slow waves in grass sickness horses: reduced frequency and increased duration

Immunohistochemistry

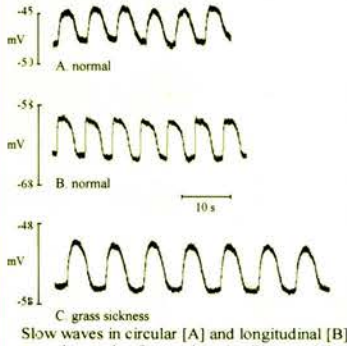
- ICC present in normal horses (Fig 6), grass sickness and obstructive bowel disease cases
- Grass sickness: ICC reduced in the myenteric plexus (MP) and circular muscle (CM) of both ileum and pelvic flexure vs normal horses (Fig 7)
- No difference in horses with strangulating small intestinal lesions
- Obstructive lesions of large colon (eg impactions, torsions and displacements): ICC reduced in MP and CM (Fig 8)

Figure 4



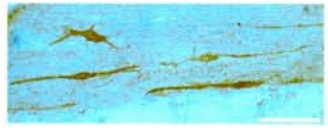
Intracellular recording from longitudinal smooth muscle of normal equine ileum showing slow waves and associated spontaneous action potentials

Figure 5



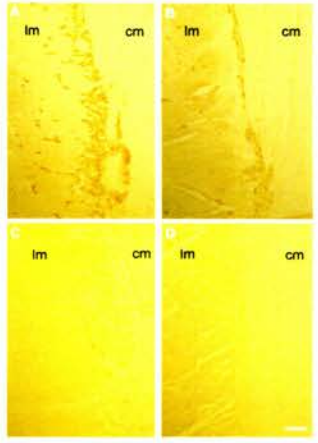
Slow waves in circular [A] and longitudinal [B] smooth muscle of normal equine ileum and the longitudinal layer of the ileum from a horse with acute grass sickness [C]. All traces in the presence of 1 µM nifedipine.

Figure 6



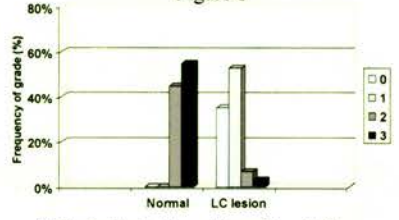
c-Kit immunoreactivity of bipolar- and stellate-shaped interstitial cells of Cajal in the circular muscle layer of normal equine small intestine. Bar = 50 µm.

Figure 7



ICC density grades in the region of the myenteric plexus of equine ileum (based on c-Kit immunoreactivity): absent (0), sparse (1), moderate (2) or abundant (3). lm= longitudinal muscle; cm= circular muscle. Bar = 100µm. A) Grade 3 in normal ileum. B) Grade 2 in normal ileum. C) Grade 1 in ileum of a horse with acute grass sickness. D) Grade 0 in ileum of a horse with acute grass sickness.

Figure 8



ICC density in the circular muscle layer of the pelvic flexure region of normal control horses (n=11) and horses with a large intestinal obstructive disorder (n=31; LC = large colon). Frequency grade (%) represents the proportion of specimens that were assigned to a particular density grade: absent (0), sparse (1), moderate (2) or abundant (3).

CONCLUSIONS

- ICC are reduced in horses with grass sickness and horses with large intestinal obstructive disorders.
- Slow wave activity was preserved in most horses with grass sickness
- The decline in ICC may be an important factor in the development of intestinal dysmotility observed in grass sickness and obstructive bowel disorders in the horse

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